

IMPROVED OXYGEN SUPPLY TO IMMOBILIZED CELLS BY *IN SITU* OXYGEN GENERATION

Olle Holst



Department of Applied Microbiology
University of Lund 1984



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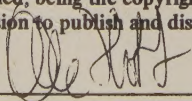
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Improved Oxygen Supply to Immobilized Cells by in situ Oxygen
Generation.

by

Olle Holst
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Abstract In processes involving immobilized aerobic cells the oxygen supply is a crucial point. This was shown for calcium alginate-immobilized <u>Gluconobacter oxydans</u> converting glycerol to dihydroxyacetone. Two methods for <u>in situ</u> oxygen generation, i.e. the production of oxygen inside the immobilization matrix, have been studied in order to improve the oxygen supply to immobilized aerobic cells. The first method is based on decomposition of hydrogen peroxide to water and oxygen. An analytical system, based on flow injection analysis, for determination of hydrogen peroxide is described. The system was used in an experimental set-up designed to maintain a constant concentration of hydrogen peroxide in the reactor. Hydrogen peroxide increased the specific productivity, but the operational stability was low. The second method is based on photolysis of water. An algae, <u>Chlorella pyrenoidosa</u>, was co-immobilized with the oxygen consumer, <u>G. oxydans</u>. The productivity of the illuminated co-immobilized preparation increased significantly compared to a preparation containing only immobilized bacteria.			
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To Lena, Martin, Gustaf, Åsa and Axel

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LIST OF PAPERS

This thesis is based on the following papers, referred to in the text by their Roman numerals.

- I Adlercreutz P, Holst O and Mattiasson B
Characterization of Gluconobacter oxydans immobilized in calcium alginate. (Submitted)
- II Holst O, Enfors S-O and Mattiasson B (1982)
Oxygenation of immobilized cells using hydrogen peroxide; a model study of Gluconobacter oxydans converting glycerol to dihydroxyacetone.
Eur J Appl Microbiol Biotechnol 14:64-68
- III Lundbäck H, Johansson G and Holst O (1983)
Determination of hydrogen peroxide for application in aerobic cell systems oxygenated via hydrogen peroxide.
Anal Chem Acta 155:47-56
- IV Holst O, Lundbäck H and Mattiasson B
Hydrogen peroxide as an oxygen source for immobilized Gluconobacter oxydans converting glycerol to dihydroxyacetone (manuscript).
- V Adlercreutz P, Holst O and Mattiasson B (1982)
Oxygen supply to immobilized cells: 2. studies on a co-immobilized algae-bacteria preparation with in situ oxygen generation.
Enzyme Microb Technol 4:395-400

INTRODUCTION

Over the past twenty years the field of immobilized biocatalysts has developed rapidly. Many different types of catalysts of biological origin have been immobilized in a number of different ways. These catalysts include enzymes, organelles, microbial-, plant- and animal cells.

The extensive literature on immobilized cells has been reviewed by many authors, with regard to immobilization methods, immobilized species and industrial applications (e.g. Dunill 1980, Bucke 1983, Birnbaum et al. 1983, Mattiasson 1983, Rosevear 1984). Our increased knowledge concerning immobilized cells has also led to the commercialization of certain processes, especially in Japan.

The use of immobilized cells in fermentation technology introduces some important advantages when compared with both conventional fermentation and immobilized enzymes. Among these are:

- catalyst reuse
- protection against contaminations, pH changes etc.
- pure product streams making downstream processing easier
- possibility of running the process at higher dilution rates than predicted by growth rate.
- high cell densities giving higher reaction rates.

However, immobilization may also have certain drawbacks that reduce the number of applications. Among these are:

- diffusional limitations that reduce mass transfer
- intracellular products will be difficult to recover
- large substrate molecules cannot be attacked by extracellular enzymes due to sterical hindrance.

The first of the drawbacks mentioned here is especially important in processes requiring oxygen as this substrate has low solubility in water and aqueous media.

This thesis focuses on the problem of oxygen supply to immobilized cells. Two different methods for generating oxygen inside the immobilization matrix have been investigated. In situ oxygen generation is based on the use of compounds with high or infinite solubility in aqueous media as oxygen precursors. The precursor will be subsequently decomposed in the immediate vicinity of the oxygen consumer.

The first oxygen precursor examined is hydrogen peroxide. This molecule is decomposed by catalase to oxygen and water in the immobilized preparation. The second oxygen precursor is water photolysed by illuminated algae which are co-immobilized with the oxygen consumer.

As a model system for evaluating these methods the conversion of glycerol to dihydroxyacetone by acetic acid bacteria, Gluconobacter oxydans, immobilized in calcium alginate was used.

A ACETIC ACID BACTERIA

Acetic acid bacteria have a rather unique ability to perform incomplete oxidations of organic compounds in the presence of oxygen. This makes them interesting from an industrial point of view (Leisinger and Muller 1967).

This part of the review will briefly outline their taxonomy and metabolism to give a background as to their potential industrial application.

Acetic acid bacteria occur widely in nature, mainly on surfaces of plants and flowers. They develop as secondary flora on decaying fruits and flowers utilizing ethanol produced from sugars by different yeasts. They are also found in various alcoholic beverages.

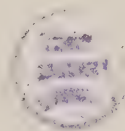
According to Bergey's Manual of Systematic Bacteriology, acetic acid bacteria belong to the family Acetobacteraceae (De Ley et al. 1984).

The family consists of two genera, Acetobacter and Gluconobacter. These two genera are recognized by their ability to oxidize ethanol to acetic acid. Recently "Acetobacter aurantius", also with this ability, was placed in a genus of its own, Frateuria. This genus was placed in the family Pseudomonadaceae (Swings et al. 1980).

The genus Frateuria contains only one species, Frateuria aurantia, as do Gluconobacter. Gluconobacter oxydans exists as five different strains, all recognized in the Approved List of Bacterial Names (Skerman et al. 1980).

The taxonomy of acetic acid bacteria has been discussed for many years and different names have been used. Gluconobacter has formerly been called both Acetomonas and Acetobacter (Asai 1968).

In this review, the name used by the author of the paper referred to has been used.



In Table 1 some of the criteria used for differentiating the genera Gluconobacter, Acetobacter and Frateuria are given.

Table 1. Characteristics for differentiating the genera Gluconobacter, Acetobacter and Frateuria (from Swings et al. 1984)

<u>Characteristics</u>	<u>Genera</u>		
	<u>Gluconobacter</u>	<u>Acetobacter</u>	<u>Frateuria</u>
Flagells in motile strains			
Polar	-	+	+
Peritrichous	+	-	-
Overoxidation of ethanol	-	+	-
Oxidation of DL-lactate to			
CO ₂ and H ₂ O	-	+	+
Formation of H ₂ S	+	-	-
Carbon sources for growth:			
D-mannose and L-arabinose	-	-	+
Types of ubiquinone formed			
Q8	-	-	+
Q9	+	D	-
Q10	-	D	-

Acetic acid bacteria have been used in industrial microbiology for more than a century. The main process based on these bacteria is the manufacture of vinegar (Ebner 1982). The industrial interest in acetic acid bacteria stems from their ability to perform incomplete oxidations of a number of different types of substances. Some of the major groups of chemicals oxidized by acetic acid bacteria are listed in Table 2.

Table 2. Compounds oxidized by acetic acid bacteria (from Leisinger and Muller 1967).

<u>Type of compound</u>	<u>Example</u>
Alcohols	
primary	ethanol
secondary	1,2 propane diol
polyols	glycerol
cyclitols	cyclohexane diols
Sugar	glucose
Organic acids	lactic acid

Many of these transformations follow Bertrand-Hudson's rule (Asai 1968). This rule states that "polyols with two secondary hydroxyl groups adjacent to the primary alcohol group are oxidized to the corresponding ketoses (Fig. 1)". This oxidation is carried out by Glucobacter oxydans and most of the species in the genus Acetobacter.

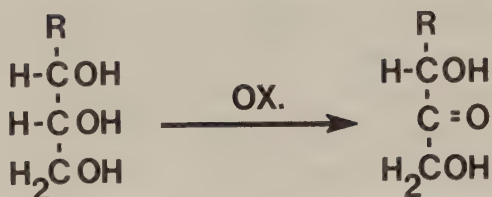


Fig. 1. Bertrand-Hudson's rule.

The simplest of these reactions is the oxidation of glycerol to dihydroxyacetone. This conversion has been studied throughout this work (Papers I, II, IV, V).

Many examples of substrates and products in conversions carried out by acetic acid bacteria are found in the literature. Some of these are of great industrial importance and processes based on the reactions have been commercialized. Others are of purely academic interest but it should be pointed out that many of these substances are difficult, if not impossible, to synthesize through organic chemistry so microbial transformation is an easy way to obtain them. In fact, organic chemists once used acetic acid bacteria for preparative purposes. It should be mentioned that not only reactions according to Bertrand-Hudson's rule are performed.

Many of the conversions carried out by acetic acid bacteria are characterized by their high yield, often as high as 90-95%.

The biochemical activities of acetic acid bacteria have been reviewed by several authors (eg. Kersters and De Ley 1962, De Ley and Kersters 1964, Asai 1968, Gottschalk 1979).

The metabolism in acetic acid bacteria is reviewed by several authors (e.g. Asai 1968, Gottschalk 1979).

The main feature of the metabolism of Gluconobacter is that a complete TCA cycle is lacking and that sugars are metabolised via the hexose monophosphate pathway.

When Acetobacter suboxydans was grown on a rich medium containing glycerol, cells harvested in the exponential phase differed from cells harvested in the stationary phase when observed in a light microscope. At the ends of the rod-shaped cells harvested in the stationary phase, dark areas were seen. Electron microscope studies revealed formation of membrane structures when the culture entered the stationary phase (Batzing and Claus 1973).

As the oxidation of polyols by Gluconobacter oxydans is carried out by membrane associated enzymes, Claus et al (1975) investigated the effect of the membrane formation on the ability of the microorganism to oxidize glycerol. They found that the cells containing intracytoplasmic membranes were 100% more active than the cells without these membranes. Further studies revealed that the oxidation rate of mannitol, glucose, erythritol and inositol also increased (White and Claus 1982).

The formation of intracytoplasmatic membranes at the entry of the stationary phase was accompanied by a change in cell size, cell density and amount of lipids in the cells (Heefner and Claus 1976). Even though the lipid content of the cells increased by 60% no drastic changes in the lipid and fatty acid composition occurred (Heefner and Claus 1978). These studies illustrate the importance of the cultivation procedure when growing microorganisms for immobilization.

In this work (See Paper II) cells were grown on glycerol as a carbon source and harvested in the early stationary phase.

B DIHYDROXYACETONE

Dihydroxyacetone (DHA) (1,3-dihydroxy-2-propanone) is a compound currently produced by microbial means by oxidizing glycerol with acetic acid bacteria. The process is described in the literature (Green et al. 1961, Green 1967) and is also covered by certain patents (e.g. Green 1960, Charney 1978).

The main interest in DHA, from an industrial point of view, is its usage in the cosmetic industry, where it is used, for example, as an artificial suntanning agent. DHA reacts with amino groups in the proteins of the skin, yielding brownish products, similar to Mallaird-reactions (Meybeck 1977, Kawashima et al. 1980).

Other applications have been discussed. When Baxter Laboratories commercialized their microbial process for the production of DHA in the late 50's (Anon 1960a,b) many other potential uses were suggested. Among these were building blocks for pharmaceuticals, dyes, food grade emulsifiers, plasticizers and resins. However, it does not seem as if any of these ideas have been realized to any great extent.

Another potential application, first suggested some ten years ago, is the use of DHA in the preservation of blood (Brake and Deindoerfer 1973). DHA helps to maintain 2,3-diphosphoglycerate (2,3-DPG) concentrations in stored blood. The 2,3-DPG concentration is important to red blood cell function since it is a modulator of the hemoglobin-oxygen release reaction (e.g. Moore et al. 1980).

The price of dihydroxyacetone is currently (Sept 1984) about 350 SEK/kg. This should be compared with the low price of the substrate, glycerol, in the conversion. Glycerol costs about 15 SEK/kg (Anon 1984).

Hydrogen peroxide costs 12 SEK/kg (Anon 1984). However, the price is dependent upon the quality used.

C IMMOBILIZED CELLS

Immobilization methods

A number of different techniques have been used for the immobilization of enzymes, organelles and whole cells. The different methods are summarized in Table 3. Further information on the methods are found in certain recent reviews on immobilized biocatalysts (e.g. Birnbaum et al 1983, Buchholz 1979, Mattiasson 1983, Nilsson 1983).

Table 3 Immobilization techniques

<u>Method</u>	<u>Reference</u>
Adsorption	Ash (1979), Nilsson (1984)
Covalent coupling	Jack and Zajic (1977)
Crosslinking	Chibata et al. (1974)
Entrapment	Hackel et al (1975)
Encapsulation	Mohan and Li (1975)
Two phase systems	Hahn-Hägerdal et al (1981), Lilly (1982)
Membrane reactors	Mattiasson et al (1981)

Of the methods listed in Table 3, entrapment is the most popular when dealing with whole cells. Among the different techniques of entrapment, the precipitation in alginate gels is probably, due to its simplicity, the most common. As alginate entrapment has been used for immobilization in this work (Papers I, II, IV, V) a short comment on this technique follows.

Alginic acid is a natural polymer consisting of mannuronic and guluronic acid (Fig. 2)

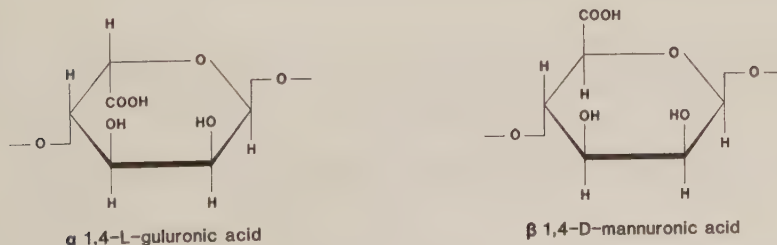


Fig. 2. Chemical structures of the building blocks of alginate.

The alginic acid is obtained from different seaweeds such as Laminaria hyperborea and Macrocystis pyrifera. The contents of mannuronic and guluronic acids, of importance to the properties of alginate, vary between different species and also between different parts of the plants.

The uronic acids contain carboxylic groups which react with divalent ions, such as calcium, and alginate gel precipitates.

In this way beads are easily formed by extruding a suspension of sodium alginate and cells through a needle and allowing the drops formed to fall into a solution of calcium chloride.

Precipitation of alginate gels as a way of immobilizing microorganisms was first employed by Hackel and co-workers for inclusion of Candida tropicalis oxidizing phenol (Hackel et al.1975).

Kierstan and Bucke (1977) used alginate to immobilize whole cells, organelles, as well as enzymes.

Further examples of immobilizations in alginate are given by Mattiasson (1983).

The mechanical properties of alginate gels depend on the chemical composition of the alginate, the concentration of calcium chloride, the concentration of alginate and the number of microorganisms.

If properly prepared, with respect to the parameters mentioned above, alginate gels have suitable properties for use in packed columns (Cheetham 1979, Cheetham et al.1979) and bubble columns (van Ginkel et al.1983, paper I). A stirred tank can also be used if stirring is not vigorous.

Apart from the simplicity the mildness of the method is a great advantage. No loss of activity was noted when G. oxydans was immobilized in calcium alginate and subsequently released (Paper I).

A major drawback with the method is that medium containing calcium binding ions, e.g. phosphate cannot be used unless the gel is stabilized. Covalent stabilization of alginate gels using e.g. glutaraldehyde and polyethyleneimine has been proposed (Birnbaum et al.1981).

However, it should be pointed out that the possibility of solubilization of the beads is also an advantage when dealing with fundamental studies on immobilized cells since dissolution is easily made.

Characterization of immobilized biocatalysts

Since the first publication concerning immobilized cells appeared in the literature in the mid 60's (Mosbach and Mosbach 1966) an ever increasing number of papers on the subject have been published. The quality of the papers have varied greatly, making interpretation and evaluation difficult, if not impossible.

The first attempt to find a remedy for this problem was made at the first Enzyme Engineering Conference in 1971 (Sundaram et al. 1972).

At this time some general guidelines for the characterization of immobilized biocatalysts (enzymes) were given. Later a new effort was made to draw attention to the problem with the publishing of "Characterization of Immobilized Biocatalysts" (Buchholtz 1979), which considered both immobilized enzymes and cells.

New guidelines were worked out by the "Working party on immobilized biocatalysts" within the European Federation of Biotechnology (EFB) in 1983 (European Federation of Biotechnology 1983). These guidelines were accompanied by an example concerning Nitrosomonas europaea immobilized in calcium alginate for nitrification of waste water (van Ginkel et al 1983). The recommendations of the working party of EFB relate to particular biocatalysts for the use in preparative and industrial applications. Additional guidelines for other areas, e.g. analytical and biomedical applications, have to be worked out by experts in these fields.

Generally speaking, the characterization should include chemical, physical/mechanical and catalytic descriptions of the biocatalyst. The recommendations contain a list of desirable minimum requirements for description of an immobilized biocatalyst. The list is briefly outlined below.

1. General description with reaction scheme, enzyme/microorganism, carrier type and method of immobilization.
2. Preparation of the biocatalyst with reaction conditions, dry weight yield etc.
3. Physical/chemical characterization with biocatalyst shape, swelling behaviour, compression in packed bed, abrasion in stirred tank etc.

4. Immobilized biocatalyst kinetics with effects of pH, buffer, diffusional limitations, degree of conversion vs residence time, storage stability, operational stability, etc.

In Paper I Gluconobacter oxydans ATCC 621 immobilized in calcium alginate for the conversion of glycerol to dihydroxyacetone is characterized according to these guidelines. The most important results are summarized in Table 4. Experimental details are given in Paper I.

Table 4. Characterization of calcium alginate immobilized Gluconobacter oxydans

	free cells	immobilized cells
Optimal pH	5	5
Optimal temperature (°C)	40	40
Storage stability (4°C) $t_{\frac{1}{2}}$ (days)	> 68	> 68
Operational stability $t_{\frac{1}{2}}$ (days)	ND	5
Maximal conversion rate (mmol DHA/g dw·h)	86	20 ¹⁾

1) With oxygen as oxygen source

ND = not determined

Diffusion in alginate gels

The catalytic performance of an immobilized cell preparation is to a great extent influenced by mass transfer phenomena. Substrate and product should be transported to and from the reaction site. This transport is accomplished in two ways, convection and diffusion. If the transport of substrate and/or product is slow this will limit the rate of reaction. In this context two different phenomena must be taken into account:

1. Firstly, the substrate must be transported from the bulk liquid to the surface of the particle. This process is carried out by convection except for a thin layer outside the particle where diffusion takes over. This layer is known as the Nernst diffusion layer.
2. Secondly, the substrate must be transported inside the matrix to the catalytic site. This intraparticle mass transfer is due to diffusion.

These external and internal mass transports, are of importance both for substrate and product.

As pointed out above the external mass transfer is mainly carried out by convection in the bulk liquid. However, near the surface of the particle a layer, the Nernst diffusion layer, is formed where transport only occurs via diffusion.

Direct measurement of the thickness of this layer is difficult to carry out. Kasche and Kuhlmann (1980) succeeded, however, by using an oxygen microprobe in a system with glucose oxidase (EC 1.1.3.4) and catalase (EC 1.11.1.6) immobilized in polyacrylamide particles with a diameter of 600 μm . They studied the influence of different relative velocities between the solvent and the particles. They found that the thickness of the layer was approximately the same as the radius of the particle when the flow rate was zero. The thickness was not negligible even at the highest flow rates applied ($7.5 \times 10^{-4} \text{ ms}^{-1}$). Their results show that the thickness of the Nernst layer cannot be neglected when designing a system with immobilized biocatalysts.

The internal mass transfer of substrates and products governs the reaction rate in processes with immobilized cells if external mass transfer is minimized. The diffusion of molecules in gels is, among

other things, dependent on the pore size of the matrix. A few reports in the literature discuss this with reference to Ca-alginate. These reports will be summarized below.

Klein et al. (1983) used size exclusion chromatography (SEC) to study the pore size in Ca-alginate gels. Small beads (10-120 μm) were packed in columns and solutions containing dextrans of different molecular weights were pumped through. Molecular weights in the range 18 400 - 5000 000 g/mole were used. As a totally permeated substance glucose was used. Enzymes of different molecular weights were also used. Glucose oxidase (GOD) and catalase (CAT) in gluconic acid production with immobilized Aspergillus niger were studied with respect to their diffusivity in alginate. The pore size was found through SEC to be in the order of 7 - 17 nm, depending upon the type of alginate that was used. GOD, with an approximate size of 8 nm, was not immobilized in the gel.

Tanaka and co-workers (1984) studied the diffusion of different substrates in Ca-alginate beads. Diffusion into and from the beads was investigated by preparing beads with and without molecules of different sizes and following the respective increase and the decrease in the surrounding solution. They estimated that at least small molecules, i.e. less than 2×10^4 daltons, diffuse as in water.

These findings contradicts the result from Kierstan and Bucke (1977) who stated that glucose oxidase (MW 1.54×10^5) did not diffuse in alginate gels. The reason for this might be the difference in the structure of molecules, such as shape and charge. Different types of alginate could also be a reason for this inconsistency. It is also interesting to note that Klein et al. (1983) found that GOD diffuses out of alginate beads.

The concentration of alginate in the beads affects the diffusion of large molecules, while the CaCl_2 -concentration in the preparation did not influence the diffusivity of large molecules to any great extent.

The main conclusion to be drawn from these studies is that only large particles, such as whole microbial cells, plant cells, animal cells and molecules having very high molecular weights, are completely immobilized in Ca-alginate. Molecules with a molecular weight below 2×10^4 diffuse freely, i.e. they have a diffusion coefficient not unlike that in water, in alginate gels. This rather approximate molecular weight is not general for alginate gels as several different types of alginate

exist. It must also be pointed out that not only the molecular weight alone governs the diffusivity but also the shape of the molecules. The charge of the molecules is also of importance.

These statements regarding the molecular weights are contradicted by the fact that oxygen has a significantly lower diffusivity in alginate gels than in water. Adlercreutz (in manuscript) found that the diffusivity of oxygen in alginate gels was 85% of that in water. Similar results were reported for barium alginate gels (Hiemstra et al. 1983) and agar (Sato and Toda 1983).

It should be pointed out that the effective diffusivity in polymeric matrixes used for immobilization depends on the amount of biocatalyst immobilized (Klein and Washausen 1979).

D OXYGENATION OF IMMOBILIZED CELLS

As pointed out above, substrate diffusion is often the rate limiting step in systems with immobilized cells. This is especially so for substrates, such as oxygen, with low solubility in the medium used.

The supply of oxygen in aerobic processes is a fundamental problem facing the fermentation technologists dealing with conventional fermentors. If the problem is great in stirred tanks with suspended cells, it is even greater in systems with immobilized cells. This is partly due to the diffusion limitations introduced by the matrix and partly due to the use of high cell densities which increase the need for oxygen.

A simple calculation (after Enfors and Mattiasson 1983) might serve as an illustration of the difficulties of supplying oxygen to a packed bed with immobilized aerobic cells. Imagine a process where glucose is oxidized to carbon dioxide and water. According to stoichiometry this requires 6 moles of oxygen for every mole of glucose. If the column has a volume of one liter and is filled with 500 g gel containing 20 g of cells with a specific activity of 1 g glucose/g cells $\cdot$ h, this packed bed will have a total glucose consumption rate of 20 g/h. The oxygen requirement will be 667 mmol/h. The solubility of oxygen in water is approximately 0.25 mM. If recirculated aqueous media, which have a solubility of oxygen in the same order as water, are to be used, a flow rate of 2700 l/h must be applied. Even this flow rate will probably not be sufficient since a concentration of oxygen above zero at the outlet is necessary in maintaining the driving force for in order to keep up an acceptable diffusion rate of oxygen.

Complete conversion of glucose by immobilized cells is only tentative but even if only a one step oxidation is supposed, thus considerably lowering the oxygen requirement, very high flow rates must be used.

If aerated stirred tanks are used, similar problems arise. The mechanical stability of the matrix will limit the possibility of using high stirrer speeds to increase the oxygen transfer rate. As one advantage of immobilized cells is that high cell densities can be used in continuous processes, high demands must be made on the oxygen transfer capacity of the bioreactor.

To find a remedy for the problem of oxygenation of immobilized aerobic cells several different attempts based on entirely different principles have been made. Some of these studies illustrating different principles, are listed in Table 5. Further elaborations are made below.

Increased oxygen partial pressure

Oxygen supply to a conventional bioreactor can be substantially improved if pure oxygen instead of air is sparged through the fermentor. However, this is a rather expensive method and is probably only worthwhile using in old plants with a low oxygen transfer capacity. High oxygen partial pressure might also be deleterious to some microorganisms. It has, for example, been used as a means of enhancing oxygenation in batch cultures of Gluconobacter melanogenus converting glycerol to dihydroxyacetone (Flickinger and Perlman 1977). It was found that the production of dihydroxyacetone continued at the same rate as when air was used but that growth was impaired. The specific growth rate decreased from 0.23 h^{-1} to 0.06 h^{-1} , indicating the negative effects of oxygen.

Similar results were obtained by Kustova and co-workers (1979) who investigated the effects of aeration on growing and resting cells of Gluconobacter oxydans oxidizing glycerol to dihydroxyacetone. The productivity of resting cells increased drastically when aeration was increased. The growth rate was not affected by the aeration rate in these experiments where only air was used.

Consequently, as the growth rate of acetic acid bacteria is impaired by pure oxygen and the productivity of resting cells improved by pure oxygen, the use of pure oxygen for the oxygenation of immobilized resting cells seems favourable. This has also been applied. In Paper II, where a packed column was fed with air saturated and oxygen saturated medium an improvement was noted in the latter case. Makhotkina et al. (1981) used pure oxygen for converting glycerol by Gluconobacter oxydans immobilized in polyacrylamide. The specific activity of the immobilized cells increased by a factor of 1.8 compared to when air was used as the oxygen source. When pure oxygen is used, negative effects on the microorganism may appear. If this were the case the operational stability would be different in systems aerated with air or oxygen. However, this was not examined in the above mentioned studies.

Table 5. Examples of attempts to increase oxygen supply in systems with immobilized biocatalysts.

<u>Principle</u>	<u>Organism/enzyme</u>	<u>Substrate</u>	<u>Product</u>	<u>Reference</u>
Increased oxygen partial pressure	G. oxydans "-	glycerol "-	dihydroxyacetone "-	Makhotkina et al. (1981) Paper II
Increased solubility				
Perfluorochemicals	"-	"-	"-	Adlercreutz and Mattiasson (1982a)
Organic solvents	Nocardia sp.	cholesterol	cholestenone	Buckland et al. (1975)
Decomposition of hydrogen peroxide	G. oxydans	glycerol	dihydroxyacetone	Papers II, IV
Photolysis of water	Chlorella pyrenoidosa/ G. oxydans Chlorella vulgaris/ Providencia sp.	glycerol aminoacids	dihydroxyacetone α -ketoacids	Paper V Wikström et al. (1982)
Electrolysis of water	glucose oxidase	glucose	gluconic acid	Enfors (1981)
Artificial electron acceptors	G. oxydans Arthrobacter simplex	glycerol cortisol	dihydroxyacetone prednisolone	Adlercreutz and Mattiasson (1984) Ohlson et al. (1978)

Increased oxygen solubility

As depicted by Fick's first law of diffusion the driving force for diffusion is the difference in concentration. Thus, a modification of the medium so that it can contain more oxygen would be a way of improving the oxygen supply to immobilized cells. This has been done for steroid conversions where organic solvents have been used in order to increase the solubility of both steroids and oxygen (Buckland et al. 1975). Organic solvents are often harmful to microorganisms and are, for example, used to permeabilize cell membranes (Felix 1982). If this method is to be used it is necessary to use organisms that are either tolerant towards organic solvents or at least have enzyme systems of interest that are stable in the presence of the solvent.

The use of organic solvents in biocatalytic systems has recently been reviewed by Lilly (1982).

The solubility of oxygen in the medium can also be increased by adding perfluorochemicals to the medium. Perfluorochemicals are hydrocarbons where all the hydrogen atoms have been substituted with fluor. Some examples are given in Figure 3. These compounds are nonpolar, which makes it necessary to add an emulsifying agent to the substrate. They are also biocompatible and have been used, at least in the laboratory, as blood substitutes in both animals (Riess and Leblanc 1978) and humans (e.g. Maugh 1979). The use of perfluorochemicals is based on their ability to dissolve high concentrations of gases, such as oxygen and carbon dioxide.

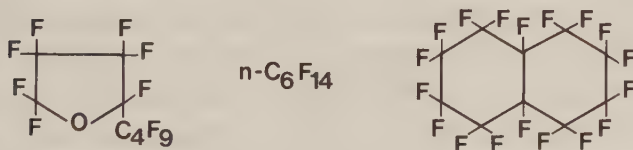


Fig. 3. Examples of chemical structures of perfluorochemicals.

Perfluorochemicals have been used as "oxygen carriers" in systems with immobilized biocatalysts.

The perfluorochemical FC-72 was used for oxygenating immobilized aerobic cells oxidizing glycerol to dihydroxyacetone (Adlercreutz and Mattiasson 1982a). A marked enhancement in productivity was noted. An important factor when using additives such as these compounds is the ability of the microorganism to withstand them.

Operational stability for Gluconobacter oxydans immobilized in calcium alginate for conversion of glycerol was investigated over a period of 160 h and no significant decrease in productivity was noted. Immobilized cells were also incubated in the presence of FC-72 emulsions without glycerol and the number of viable cells was followed for 12 days. Compared with incubation in buffer solution, with glycerol, no significant differences in viable counts was noted.

Thus, it appears that perfluorochemicals are well suited for improving oxygen supply to immobilized aerobic cells since they increase the solubility of oxygen significantly and thereby increase the productivity. They are biocompatible to the extent that no deleterious effects were noted.

Oxygen carriers

Another way of increasing the amount of oxygen in the medium is to use molecules that bind oxygen to specific sites. One such molecule is hemoglobin. This substance was used as the oxygen carrier for immobilized whole cells (Adlercreutz and Mattiasson 1982a). Enhanced productivity was observed in a system with Gluconobacter oxydans, immobilized in calcium alginate, oxidizing glycerol. The medium containing hemoglobin was recirculated, after aeration, into a packed column. The process was studied at different pH and it was found that hemoglobin was suitable as a transport molecule at pH's above 6. At pH 5 irreversible oxidation of hemoglobin to methemoglobin occurred.

Proteolytic activity of the immobilized cells must also be avoided in order to prevent destruction of the carrier molecule.

Decomposition of hydrogen peroxide

Oxygen can be supplied to biocatalysts by catalytic decomposition of hydrogen peroxide. This has been used for fermentors (e.g. Schlegel 1976, 1977), immobilized enzymes (e.g. Lawny et al. 1975), immobilized non viable cells (Hartmeier and Döppner 1983) and immobilized viable cells (Papers II, IV). The catalytic decomposition can be made with inorganic catalysts co-immobilized with the biocatalyst such as activated carbon (Szwajcer et al. 1982) or external (Ibrahim and Schlegel 1980a) as well as internal catalase (Papers II, IV).

The following part of the review will give some examples of this approach and discuss the advantages and disadvantages of the method.

Schlegel (1976, 1977) cultivated Acinetobacter calcoaceticus, Alcaligenes eutrophus, Candida oleophila, Paracoccus denitrificans, Pseudomonas putida and Saccharomyces cerevisiae with hydrogen peroxide as an oxygen source. In these studies catalase from bovine liver was added to shaker flasks and diluted solutions of hydrogen peroxide were pumped in continuously. The atmosphere in the flasks was nitrogen. All of the microorganisms tested exhibited growth, as measured with optical density, but some were affected by the peroxide resulting in impaired growth.

It is interesting to note that it was claimed that the intracellular catalase of the microorganisms does not contribute to the cleavage of the peroxide.

The early works by Schlegel (1976, 1977) concerned only low cell densities with rather low oxygen requirements. Further studies were performed by Ibrahim and Schlegel (1980a) to investigate the effects of hydrogen peroxide on bacterial suspensions of high cell densities.

As a model system Alcaligenes eutrophus was studied, cultivated in a fermentor supplied with catalase from bovine liver. The dosage of hydrogen peroxide was controlled by measuring the amount of dissolved oxygen. Although growth was normal in the early exponential phase it was found that growth was impaired at high cell densities (5-20 g/L). The reason for this was that even though catalase was present all the hydrogen peroxide added was not decomposed. The incomplete decomposition of hydrogen peroxide was due to the low affinity of catalase towards hydrogen peroxide and the rapid inactivation of the enzyme during

the addition of hydrogen peroxide (Ibrahim and Schlegel 1980b). The hydrogen peroxide present in the fermentor affected the activity of a number of enzymes in the microorganisms in a negative way. The only enzyme that showed an increase in activity was catalase. The microorganisms also formed giant cells and their ability to excrete siderophores was affected negatively. The rate of synthesis of macromolecules such as proteins, RNA and DNA was also diminished.

If hydrogen peroxide should be used as the oxygen source in fermentors a more efficient enzyme for cleaving hydrogen peroxide must be found. Nies and Schlegel (1984) were succesful when they found a catalase from Escherichia coli which exhibited certain unique properties. It was shown that the catalase from E. coli was better than the catalase from bovine liver for oxygen supply to cell suspensions using hydrogen peroxide. The main difference between these two catalases, from a practical point of view, is that when bovine liver catalase is used, toxic amounts of hydrogen peroxide are still present in the fermentor. Mathematical models and experiments with open systems show that this is not the case for catalase from E. coli, although experiments in fermentors remain to be done.

Hydrogen peroxide has also been used as an oxygen source for oxygenating activated sludge (e.g. Houtmeyers et al. 1977). A temporary decrease in COD reduction, an increase in the catalase activity of the sludge and a depression of the nitrification was noted. The increase in catalase activity was believed to be a result of stimulating of the catalase synthesis.

The use of hydrogen peroxide as the oxygen supplement and deodorizing agent in waste water treatment has recently been reviewed by Schwab (1984).

The productivity of systems with immobilized enzymes have also been improved with hydrogen peroxide.

Lawny et al. (1975) added hydrogen peroxide to a system with immobilized glucose oxidase and catalase. Enhancement of oxidase reactor productivity was noted by Fink (1977) who worked with aminoacid oxidase and catalase immobilized in glass. Hartmeier and Tegge (1979) also used glucose oxidase and catalase bound to gelatin particles by glutardialdehyde. Later, Hartmeier and Döppner (1983) coupled catalase

directly to mycelia of Aspergillus niger exhibiting glucose oxidase activity in order to improve the stability of the preparation against hydrogen peroxide.

Submerged attached cells, nitrifying ammonia have also been oxygenated via hydrogen peroxide (Yahi et al. 1982).

Hydrogen peroxide has also been used as the oxygen source for Methylococcus thermophilus adsorbed on charcoal for removing methane from drain water in coal mines (Malashenko et al. 1983).

In Paper II resting cells of Gluconobacter oxydans immobilized in Ca-alginate for converting glycerol to dihydroxyacetone were oxygenated via hydrogen peroxide. A drastic increase in productivity was noted in a packed column. However, the half-life of the preparation was only 30 h.

When hydrogen peroxide is used as a source of oxygen for microorganisms (biocatalysts) two aspects must be considered. Firstly, the aeration of the fermentors has a dual function: oxygen should be supplied and carbon dioxide should be removed. Only the first of these functions is accomplished using hydrogen peroxide. Carbon dioxide has several negative effects on microorganisms (Jones and Greenfield 1982) and thus high concentrations should be avoided. With respect to immobilized cells the removal of carbon dioxide is also important from a mechanical point of view. Bubbles of carbon dioxide will disrupt the matrix and increase cell leakage. This has been discussed in detail by Krouwel (1982).

Secondly, the oxygen concentration in a normally aerated fermentor is always low, i.e. never higher than the concentration depicted by the partial pressure of oxygen in the gas phase. In practice it is often lower due to the consumption by the microorganisms, which is balanced by the oxygen transfer rate. When hydrogen peroxide is used the oxygen concentration may rise considerably if the oxygen production by catalytic cleavage of hydrogen peroxide exceeds the oxygen consumption rate. High partial pressure of oxygen may be deleterious to microorganisms.

Consequently, the dosage of hydrogen peroxide must be controlled. This can be done by measuring the dissolved oxygen and keeping this at a desirable level by adding peroxide. This approach was used by Ibrahim and Schlegel (1980a). The desirable level should then be that which gives optimal growth (if in a fermentor) or the optimal production rate (if immobilized biocatalysts).

The use of dissolved oxygen values for controlling the dosage of hydrogen peroxide has a severe drawback. As the activity of the catalase slowly decreases during the process an increased concentration of hydrogen peroxide is necessary in maintaining the desired oxygen production rate. This will probably cause a rapid deactivation of the catalase as well as the other biocatalyst.

Another approach is to measure the amount of hydrogen peroxide present and determine the level which gives the optimal growth or productivity. This approach was used in Paper IV where a flow injection analysis system was used for determining hydrogen peroxide. Simultaneous measurements of dissolved oxygen were also made.

Hydrogen peroxide also seems to be more dangerous to growing cells than to resting cells. This is an advantage when immobilized resting cells are used.

When hydrogen peroxide is used as an oxygen source negative effects on the biocatalysts are noted. Different ways of remedying this have been suggested.

Induction of catalase in the cells by growing them in the presence of hydrogen peroxide may be a way of improving the ability of the microorganism to withstand the peroxide. This has been tested for Gluconobacter oxydans (Andersson 1984). However, these experiments have not yet been successful.

The decomposition of hydrogen peroxide can be facilitated by co-immobilization with catalysts that cleave the molecule, thereby minimizing the contact between the peroxide and the biocatalyst (Duvnjak and Lilly 1976). Co-immobilization with superoxide dismutase for scavenging radicals has also been used (Tramper et al. 1978).

It is also possible to separate the cleavage of hydrogen peroxide from the biocatalyst as described by Updike et al. (1973). They used MnO_2 precipitated in membranes as a catalyst for decomposing hydrogen peroxide in order to improve oxygenation in artificial lungs.

Yet another approach has been described by Adlercreutz and Mattiasson (1984). They used hydrogen peroxide and immobilized peroxidase to regenerate hydroquinone to p-benzoquinone when the latter had been used as an artificial electron acceptor (Figure 4). By this method the biocatalyst can be separated from the peroxide.

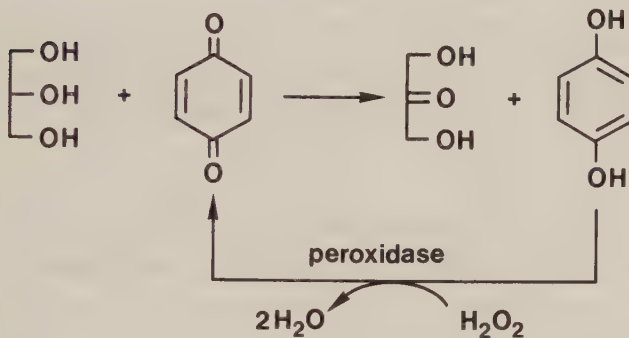


Fig. 4. Production of dihydroxyacetone aided by regenerated artificial electron acceptors.

If damage occurs methods are available to restore the activity, at least in certain cases. Couderc and Baratti (1980) studied immobilized yeast cells with methanol oxidase activity. The hydrogen peroxide formed in the enzymatic reaction inactivated the enzyme. The inactivation mechanism was proven to be the oxidation of SH-groups of methanol oxidase. Activity could be restored by treating with β -mercaptoethanol. The use of β -mercaptoethanol could also prevent deactivation.

Yet another method of regaining the activity would be to reincubate the immobilized cells in fresh medium.

Photolysis of water

One way of generating oxygen in situ is to co-immobilize the oxygen consuming biocatalyst with algae that produce oxygen upon illumination (Paper V, Wikström et al. 1982).

Prior to elaboration on these works a short comment on mixed cultures and co-immobilization is useful.

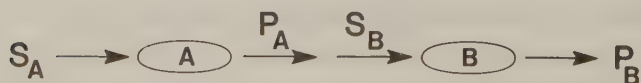
Stable mixed cultures of microorganisms occur extensively in nature and man-made environments. Lichens, a symbiosis between algae and fungi, and undefined mixed cultures in sewage plants are well-known examples. The interactions between microorganisms can be described in several different ways: parasitism, predation, commensalism etc., depending on how the microorganisms use their substrates and what occurs to the products. For discussions concerning mixed cultures, see e.g. Harrisson (1978).

Intentional co-immobilization of defined species is a rather new area not yet fully explored. The subject has recently been reviewed by Hahn-Hägerdal (1983). Immobilization of different catalytic active species in or on the same matrix/carrier adds some extra advantages to the concept of immobilization (see above). New metabolic pathways can be created providing an alternative to genetic engineering (Hahn-Hägerdal 1983). Further, the proximity effect, as shown by Mattiasson (1974) for immobilized multi-enzyme systems, is also important.

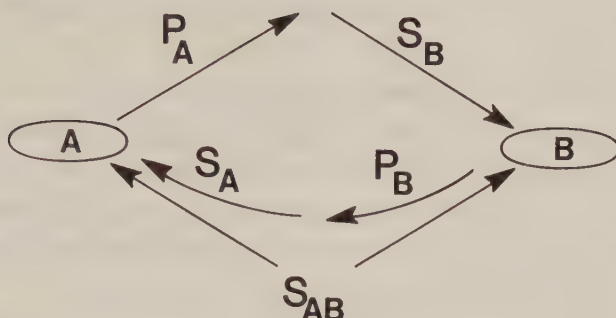
However, this proximity effect is only at work if the different species have approximately the same optima with regard to environmental parameters such as pH, temperature, etc.

For co-immobilized preparations mainly two types of interactions are of interest:

1. Commensalism - the product from one specie is used as a substrate or cofactor by the other.



2. Mutualism - mutual production of substrates or cofactors for the other specie.



A number of different possibilities exist when choosing species involved in a co-immobilization. Microorganisms (procaryotes) can be co-immobilized with other microorganisms, either procaryotes (e.g. Martin and Perlman 1976) or eucaryotes (e.g. Paper V). They can also be co-immobilized with organells (Karube et al. 1981), enzymes (Hahn- Hägerdal 1984) or inorganic catalysts (Swajcer et al. 1982). Of course other combinations are possible too.

In Paper V a commensal type of interaction is described. An algae, Chlorella pyrenoidosa, was co-immobilized with an acetic acid bacteria, Gluconobacter oxydans, in order to improve the oxygen supply. G. oxydans converted glycerol to dihydroxyacetone. When the preparation was illuminated oxygen was produced by the algae and subsequently used by the bacteria. The proximity effect is clearly seen in Fig.1 in Paper V, where separately immobilized bacteria and algae and a co-immobilized preparation are compared. A 30% higher productivity was noted when the co-immobilized preparation was used.

The possibility of using immobilized algae for producing oxygen has been investigated by Adlercreutz and Mattiasson (1982b). They used Chlorella pyrenoidosa immobilized in calcium-alginate and studied the effects of parameters such as pH, substrate (CO_3^{2-}) concentration and light intensity on the productivity of oxygen. The general conclusion to be drawn from this work is that the main limiting factor was light. Light harvesting in biotechnology raises very specific demands on the reactor design. A tubular loop reactor has been claimed to be

the most efficient bioreactor for utilizing light on a large scale using suspended cells (Pirt 1982). Extra demands must be made on the reactor when using immobilized cells due to the high percentage of cells constantly in the dark. Thin membrane layers might be a solution.

Wikström et al. (1982) investigated the co-immobilization of a bacterium, Providencia sp. and an alga, Chlorella vulgaris in beads of agarose for the production of α -ketoacids from amino acids. A considerable increase in productivity was noted. At a ratio of 2:1 of algae: bacteria, productivity, of an illuminated column was ten times higher than when running the column in the dark or without algae.

As for the co-immobilization between G. oxydans and C. pyrenoidosa (Paper V) the addition of carbonate increased productivity. No data concerning pH and temperature optima nor operational stability was given making evaluation of the system somewhat difficult.

Electrolysis of water

Decomposition of water to oxygen and hydrogen by means of electrolysis is yet another way of providing biocatalytic systems with oxygen. This has been tested with submerged cultures of Pseudomonas fluorescens (Sadoff et al. 1956). Although oxygen was generated and growth occurred in the beginning of the cultivation the growth eventually ceased. The reason for this was attributed to the electrochemical formation of hypochlorite (OCl^-). This compound was found to have deleterious effects at concentrations as low as 0.03 mg L^{-1} .

Electrolysis of water has also been applied to oxygenation of livestock wastes by Brumm et al. (1982). They found that electrolysis as a way to aerate waste water was technically feasible, but an economic evaluation of the method remains to be made.

A glucose electrode based on immobilized glucose oxidase, an oxygen requiring enzyme, with internal production of oxygen by means of electrolysis has been described (Enfors 1981).

The effects of chloride on the immobilized enzyme in this electrode was investigated by Cleland and Enfors (1983). They did not find any negative effects in fed-batch cultures of Escherichia coli in a chloride containing media.

Another probable drawback with electrolysis as a way of producing oxygen is the possibility of by-product formation due to electrochemical reactions of substrates or products. The proper choice of electrode material, electrolysis current, medium, voltage etc., is a necessity avoiding formation of unwanted by-products as well as toxic substances, such as hypochlorite, which might interact with the immobilized biocatalyst.

This approach has not yet, to my knowledge, been applied to system with immobilized whole cells.

Artificial electron acceptors

The main function of oxygen in aerobic metabolism is to take up hydrogen in oxidations, i.e. act as electron acceptor. This task can be performed by other substances which interact with the enzymes in the metabolic pathways and in the electron transport chain and pick up electrons. This ability of some molecules to act as artificial electron acceptors has been used as a way of improving the reaction rate in systems with immobilized aerobic cells.

Adlercreutz and Mattiasson (1984) used p-benzoquinone as an artificial electron acceptor in the conversion of glycerol to dihydroxyacetone by Gluconobacter oxydans immobilized in calcium alginate. When free cells were used the reaction rate was four times greater than with oxygen as the electron acceptor and when immobilized cells were used the reaction rate was 6 times higher than when oxygen was used. The operational stability was investigated over a period of 8 days. The productivity decreased from 60 to 10 mmol/g · h.

When p-benzoquinone is used as the electron acceptor hydroquinone is formed. This compound can be reoxidized to p-benzoquinone by using hydrogen peroxide and peroxidase (Fig. 4). In this way p-benzoquinone was reused 8 times without loss of efficiency (Adlercreutz and Mattiasson 1984).

P-benzoquinone is more efficient than oxygen as an electron acceptor because it is more soluble than oxygen in water and gives higher maximal reaction rates. The mechanism behind electron uptake is not known.

One drawback with the use of p-benzoquinone as an electron acceptor is that when high concentrations of p-benzoquinone and hydroquinone are at hand a 1:1 complex, quinhydrone, is formed and this precipitates. This precipitation forms a thin shell around the beads which probably decrease the reaction rate due to additional diffusion limitations.

Ohlsson et al. (1978) used menadione as a cosubstrate for Arthrobacter simplex immobilized in polyacrylamide for conversion of steroids. The activity of the immobilized cells was markedly enhanced when measuring initial rates. However, when continuous production of prednisolone was studied a drop in long-term stability was observed. It was also impossible to reincubate the preparation in the presence of menadione.

E DETERMINATION OF HYDROGEN PEROXIDE

Hydrogen peroxide has numerous applications in industry. This diversity of uses has lead to the development of a number of different principles for its determination. The different techniques are listed in Table 6.

Table 6. Techniques for determining hydrogen peroxide (after Lundbäck 1984).

Tritrimetry

Direct electrochemistry

e.g Amperometry

Chemical reaction

yielding species detected by

e.g. Spectrophotometry
Chemiluminiscence

Cleavage of hydrogen peroxide
followed by

Oxygen analysis
Thermometry

Further details concerning the methods are given by Lundbäck (1984).

In Paper III a flow injection analysis system (Ruzicka and Hansen 1981) with amperometric detection of hydrogen peroxide is described. The analytical system is illustrated in Figure 5. For comparison, two entirely different principles were also tested, one measuring the heat evolved during catalytic cleavage of hydrogen peroxide and one based on spectrophotometry in connection with peroxidase reaction. All three methods agreed well.

The injection of a sample in an unsegmented carrier flow prior to a detection device, flow injection analysis (FIA), is a rather new method developed during the last ten years (Ruzicka and Hansen 1981).

The technique is based on three events:

1. Sample injection.
2. Controlled dispersion of a well defined sample zone.
3. The exact timing of all events in each assay cycle. Thus, the method is highly reproducible even if mixing is not complete and an equilibrium not reached.

FIA provides a tool for automated sample treatment and rapid analysis. It is possible to build a complex system involving dilution, ion exchangers, dialysis etc., to tailor-make the analytical system for the desired application.

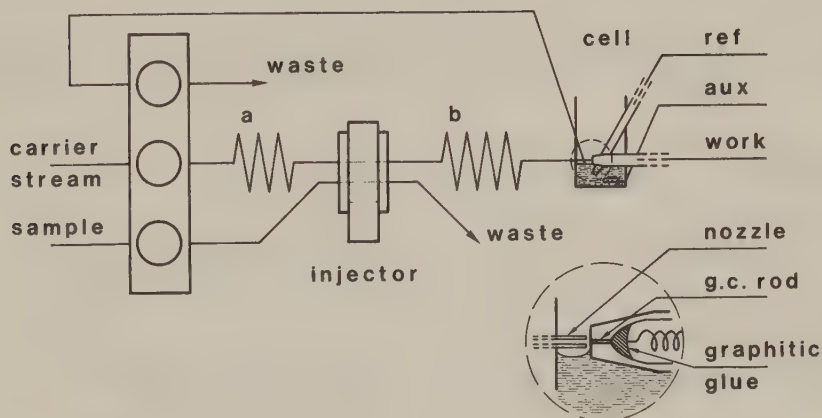


Fig. 5. Flow injection analysis system used for the determination of hydrogen peroxide. (a) Pulse damping coil; (b) dispersion coil. For further details, see Lundbäck (1984).

CONCLUDING REMARKS

A number of different applications of immobilized aerobic cells have been suggested and studied. Most reports draw the conclusion that oxygen supply is a rate limiting factor. Statements such as: " methods to supply abundant of oxygen must be developed" followed by: "If these problems can be solved, continuous production ... with immobilized cells may be industrially useful" (Nabe et al. 1979) can be found.

In this thesis, two different techniques, decomposition of hydrogen peroxide and photolysis of water, for improving the oxygen supply to immobilized cells have been investigated. Both increase the productivity of the immobilized cells, showing that methods of increasing the oxygen supply to immobilized cells exist.

Although increased specific productivity is observed when hydrogen peroxide is used as an oxygen source, the application of this method to industrial processes does not seem straightforward due to low operational stability. Further studies on different methods of increasing stability must be made. Techniques for separating the bicatalysts and the peroxide exist and this seems to be a promising possibility. The analytical technique for determining of hydrogen peroxide as described here has its obvious application in controlling the peroxide level in these systems.

When photolysis of water utilizing algae co-immobilized with bacteria is used as the method for oxygenation, the reactor design is a crucial factor.

The choice of algae is also important. The selection must be made with regard to formation of by-products, formation of toxic compounds, operational stability, need for co-factors, etc. It is also important that the product from the bacterium-catalysed reaction is not utilized by the algae. It should also be stressed that unicellular algae show great potential for use in biotechnology as light harvesting entities and producers of various chemicals and this area has much more to give (Rao and Hall 1984).

Although the model system used in this thesis exhibits a rather low operational stability the principles for oxygenation of immobilized cells seem to be useful and remain to be tested in connection with other aerobic conversions.

The methods described here, as well as those discussed in the literature must be studied further with respect to scale-up, economy etc. All the methods have their advantages and disadvantages and the one to use must be decided from case to case.

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REFERENCES

Adlercreutz P (in manus)

Adlercreutz P and Mattiasson B (1982a) Eur J Appl Microbiol Biotechnol, 16, 165

Adlercreutz P and Mattiasson B (1982b) Enzyme Microb Technol, 4, 332

Adlercreutz P and Mattiasson B (1984) Appl Microbiol Biotechnol, 20, 296

Andersson G (1984) Induction of catalase in Gluconobacter oxydans ATCC 621. Graduation Report, Dept. Appl. Microbiol., Univ. of Lund, Sweden.

Anon (1960a) Chem Eng New, Feb 22, 62

Anon (1960b) Chem Eng News, Aug 15, 54

Anon (1984) Chem Mark Rep, Sept.

Asai T (1968) Acetic acid bacteria; Classification and Biochemical Activities. University of Tokyo Press.

Ash SG (1979) In: Adhesion of microorganisms to surfaces (Ellwood DC, Melling J, Rutter P eds, p 57, Academic Press, London New York San Francisco

Batzing BL and Claus GW (1973) J Bact, 113, 1455

Birnbaum S, Larsson P-O and Mosbach K (1983) In: Solid-Phase Biochemistry: Synthetic and Analytical Aspects (Scouten WH ed), p. 679, John Wiley, New York

Birnbaum S, Pendleton, R, Larsson P-O and Mosbach K (1981) Biotechnol Lett, 3, 393

Brake JM and Deindorfer FH (1973) Transfusion, 13, 84

Brumm TJ, Steinberg MP and Day DL (1982) *Biotechnol Bioeng Symp* No 12, 327

Bucke C (1983) *Phil Trans R Soc Lond, B* 300, 369

Buchholz K ed (1979) *Characterization of Immobilized Biocatalysts*, Dechema, Monographs, Vol 84, Verlag Chemie, Weinheim and New York

Buckland BC, Dunnill P, Lilly MD (1975) *Biotechnol Bioeng*, 17, 815

Charney W (1978) US Pat 4.076.589

Chibata I, Tosa T and Sato T (1974) *Appl Microbiol*, 27, 878

Cheetham PSJ (1979) *Enzyme Microb Technol*, 1, 183

Cheetham PSJ, Blunt KW and Bucke C (1979) *Biotechnol Bioeng*, 21, 2155

Claus GW, Batzing BL, Baker CA and Goebel EM (1975) *J Bact*, 123, 1169

Cleland N and Enfors SO (1983) *Eur J Appl Microbiol Biotechnol*, 18, 141

Couderc R and Baratti J (1980) *Biotechnol Bioeng*, 22, 1155

De Ley J, Gillis M and Swings J (1984) *Acetobacteraceae*. In: *Bergey's Manual of Systematic Bacteriology* (Krieg NR and Holt JG eds) Vol 1, p 267, Williams and Wilkins, Baltimore and London

De Ley J and Kersters K (1964) *Bact Rev*, 28, 164

Dunnill P (1980) *Phil Trans R Soc Lond, B* 290, 409

Duvnjak Z and Lilly MD (1976) *Biotechnol Bioeng*, 18, 737

Ebner H (1982) *Vinegar*. In: *Prescott and Dunn's Industrial Microbiology*. (Reed G ed) p 802, AVI Publ Comp, Westport, Connecticut

Enfors SO (1981) *Enzyme Microbiol Technol*, 3, 29

Enfors S-O and Mattiasson B (1983) In: Immobilized Cells and Organelles (Mattiasson B ed) Vol II, p 41, CRC Press, Boca Raton, Fl

European Federation of Biotechnology (1983) Enzyme Microb Technol, 5, 304

Felix H (1982) Anal Biochem, 120, 221

Fink DJ (1977) Biotechnol Bioeng, 19, 1245

Flickinger MC and Perlman D (1977) Appl Environ Microbiol, 33, 706

van Ginkel CG, Tramper J, Luyben KChAM and Klapwijk A (1983) Enzyme Microb Technol, 5, 297

Gottschalk G (1979) Bacterial Metabolism, Springer-Verlag, New York Heidelberg Berlin

Green SR (1960) US Pat 2.948.658

Green SR (1967) in: Microbial Technology (Peppler HJ ed) p 360, Reinhold Publ Corp, New York.

Green SR, Whalen EA and Molokie E (1961) J Biochem Microbiol Technol Eng, 3, 351

Hackel U, Klein J, Megnet R and Wagner F (1975) Eur J Appl Microbiol, 1, 291

Hahn-Hägerdal B (1983) In: Immobilized Cells and Organelles (Mattiasson B ed) Vol II, p 79, CRC Press, Boca Raton, Fl

Hahn-Hägerdal B (1984) Biotechnol Bioeng, 26, 771

Hahn-Hägerdal B, Mattiasson B and Albertsson P-Å (1981) Biotechnol Lett, 3, 53

Harrisson DEF (1978) Adv Appl Microbiol, 24, 129

Hartmeier W and Döppner T (1983) Biotechnol Lett, 5, 743

Hartmeier W and Tegge G (1979) Starch/Stärke, 31, 348

Heefner DL and Claus GW (1976) J Bact, 125, 1163

Heefner DL and Claus GW (1978) J Bact, 134, 38

Hiemstra H, Dijkhuizen L and Harder W (1983)
Eur J Appl Microbiol Biotechnol, 18, 189

Houtmeyers J, Poffé R and Verachtert H (1977) Eur J Appl Microbiol, 4,
295

Ibrahim M and Schlegel HG (1980a) Biotechnol Bioeng, 22, 1877

Ibrahim M and Schlegel HG (1980b) Biotechnol Bioeng, 22, 1895

Jack TR and Zajic JE (1977) Biotechnol Bioeng, 19, 631

Jones RP and Greenfield PF (1982) Enzyme Microbiol Technol, 4, 210

Karube I, Matsunaga T, Otsuka T, Kayano H and Suzuki S (1981) Biochem
Biophys Acta, 637, 490

Kasche V and Kuhlmann G (1980) Enzyme Microb Technol, 2, 309

Kawashima K, Itoh H and Chibata I (1980) Agric Biol Chem, 44, 1595

Kerstens K and De Ley J (1962) Biochim Biophys Acta, 71, 311

Kierstan M and Bucke C (1977) Biotechnol Bioeng, 19, 387

Klein J, Stock J and Vorlop K-D (1983) Eur J Appl Microbiol Biotechnol
18, 86

Klein J and Washausen P (1979) In: Characterization of Immobilized
Biocatalysts (Buchholz K ed), Dechema Band 84, p 300, Verlag Chemie,
Weinheim and New York.

Krouwel PG (1982) Immobilized Cells for Continuous Solvent Production,
Thesis, Dept Chem Eng, Delft Univ, Delft, The Netherlands

- Kustova NA, Pomortseva NV, Krasilnikova TN and Nikolaev PI (1979) Prikl Biokhim Mikrobiol, 15, 51
- Lawny F, Cordonnier M and Thomas D (1975) AIChE Journal, 21, 822
- Leisinger T and Muller J (1967) Process Biochem, april, 10
- Lilly MD (1982) J Chem Tech Biotechnol, 32, 162
- Lundbäck H (1984) A Study of Some Methods Involving the Determination of Hydrogen Peroxide, in Particular in Flow Injection Analysis, Thesis, Univ. of Lund
- Malashenko YR, Karpenko VI, Muchnik FV and Romanovskaya VA (1983) Mikrobiologia, 52, 554
- Makhotkina TA, Pomortseva NV, Lomova IE and Nikolaev PI (1981) Prikl Biokhim Mikrobiol, 17, 102
- Martin CKA and Perlman D (1976) Eur J Appl Microbiol, 3, 91
- Mattiasson B (1974) Studies on Immobilized Multi-Step-Enzyme Systems, Thesis, Univ. of Lund
- Mattiasson B, ed (1983) Immobilized Cells and Organelles, Vol I and II, CRC Press, Boca Raton, Fl
- Mattiasson B, Ramstorp M, Nilsson I and Hahn-Hägerdal B (1981) Biotechnol Lett, 3, 561
- Maugh TH (1979) Science 206, 205
- Meybeck A (1977) J Soc Cosmet Chem, 28, 25
- Mohan RR and Li NN (1975) Biotechnol Bioeng, 17, 1137
- Moore GL, Ledford ME, Brummell MR and Brooks DF (1980) Transfusion, 20, 24

Mosbach K and Mosbach R (1966) Acta Chem Scand, 20, 2807

Nabe K, Izuo N, Yamada S and Chibata I (1979) Appl Environ Microbiol, 38, 1056

Nies D and Schlegel HG (1984) Biotechnol Bioeng, 26, 737

Nilsson I (1984) Influence of entrapment and surface adsorption on the conversion capacity of Pseudomonas spp., Thesis, Univ. of Lund

Nilsson K (1983) Immobilized animal and plant cells; Preparation and potential biotechnological applications, Thesis, Univ of Lund

Ohlson S, Larsson P-O and Mosbach (1978) Biotechnol Bioeng, 20, 1267

Pirt SJ (1982) J Chem Tech Biotechnol, 32, 198

Rao KK and Hall DO (1984) Trends in Biotechnology, 2, 124

Riess JG and Le Blanc M (1978) Angew Chem, 90, 654

Rosevear A (1984) J Chem Tech Biotechnol, 34B, 127

Ruzicka J and Hansen EH (1981) Flow Injection Analysis, John Wiley and Sons, New York

Sadoff HL, Halvorson HO and Finn RK (1956) Appl Microbiol, 4, 164

Sato K and Toda K (1983) J Ferment Technol, 61, 239

Schlegel HG (1976) Abstr Fifth Int Ferm Symp, Berlin, abstr 1.07, p 9

Schlegel HG (1977) Biotechnol Bioeng, 19, 413

Schwab H (1984) Forum Mikrobiologie, 7, 46

Skerman VBD, McGowan V and Sneath PHA (1980) *Int J Syst Bact*, 30, 225

Sundaram PV, Pye EK, Chang TMS, Edwards VH, Humphrey AE, Kaplan NO, Katchalski E, Levin Y, Lilly MD, Mannecke G, Mosbach K, Patchornik A, Porath J, Weetall HH and Wingard LBJr (1972) *Biotechnol Bioeng Symp*, 3, 15

Swings J, De Ley J and Gillis M (1984) *Fratueria*. In: *Bergeys's Manual of Systematic Bacteriology* (Krieg NR and Holt JG eds) Vol 1, p 210, Williams and Wilkins, Baltimore and London

Swings J, Gillis M, Kerster K, De Vos P, Gosselé F and De Ley J (1980) *Int J Syst Bact*, 30, 547

Szwajcer E, Brodelius P and Mosback K (1982) *Enzyme Microb Technol*, 4, 409

Tanaka H, Matsumura M and Veliky IA (1984) *Biotechnol Bioeng*, 26, 53

Tramper J, Müller F and Van der Plas HC (1978) *Biotechnol Bioeng*, 20, 1507

Updike SJ, Shults M and Magnuson J (1973) *J Appl Physiol*, 34, 271

White SA and Claus GW (1982) *J Bact*, 150, 934

Wikström P, Szwajcer E, Brodelius P, Nilsson K and Mosbach K (1982) *Biotechnol Lett*, 4, 153

Yahi H, Grasmick A, Elmaleh S and Faup GM (1982) *Environ Technol Lett*, 3, 281

Characterization of Gluconobacter oxydans immobilized in calcium alginate.

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Running Title: Characterization of G. oxydans in alginate gel.

Summary

Gluconobacter oxydans cells were immobilized in calcium alginate and the preparation was used for the oxidation of glycerol to dihydroxyacetone. The characterization was done according to the guidelines given by the Working Party on Immobilized Biocatalysts of the European Federation of Biotechnology. The pH optimum of the preparation was found to be 5.0 and the temperature optimum was 40°C. However, the operational stability was better at 30°C. The glycerol concentration required to obtain half the maximal reaction rate was about 5 mM for both immobilized and free cells. At low concentrations of glycerol and high concentrations of dihydroxyacetone a slight inhibition was noted. No loss of activity of the immobilized preparation was observed after storage for 68 days at +4°C. Investigation of the operational stability revealed a half-life of 5 days. Studies of the influence of particle size and cell densities as well as that of oxygen concentration revealed that the oxygen supply was the rate limiting step.

Introduction

The acetic acid bacterium Gluconobacter oxydans (ATCC 621) is able to perform incomplete oxidations of many different substrates. Several of the products formed are of commercial interest. The bacteria can, for example, oxidize glycerol to dihydroxyacetone (DHA) (Scheme 1). This reaction is done industrially with conventional fermentation technology (Green 1967). The use of immobilized cells would confer several advantages. However, when immobilized cells are used, oxygen transfer from the bulk phase to the cells in the matrix limits the reaction rate (e.g. Tramper et al 1983). Immobilized G. oxydans cells have been previously used as a model system to evaluate different methods of increasing the oxygen supply to immobilized cells (Holst et al. 1982, Adlercreutz et al. 1982, Adlercreutz and Mattiasson 1982, Adlercreutz and Mattiasson, 1984).

Some papers concerning the production of DHA with immobilized cells have already been published. Chibata and coworkers investigated the conversion of glycerol to DHA by Acetobacter xylinum immobilized in polyacrylamide gel (Nabe et al. 1979). The same conversion was studied by Makhotkina et al. (1981) with G. oxydans immobilized in polyacrylamide gel. Data on the use of this reaction as a model system when evaluating the use of hydrogen peroxide as an oxygen source for immobilized cells can be found in Holst et al. (1982).

The properties of G. oxydans immobilized in calcium alginate gel are presented here as determined according to the guidelines of the Working Party on Immobilized Biocatalysts of the European Federation of Biotechnology (European Federation of Biotechnology 1983).

Materials and Methods

Chemicals

Sodium alginate was purchased from Sigma (Type IV Lot No. 111F-0049) Dihydroxyacetone (98%) was supplied by Merck, Darmstadt, FRG. All other chemicals were of analytical grade and obtained from commercial sources.

Organism

The bacteria Gluconobacter oxydans (ATCC 621) were cultivated as previously described (Holst et al. 1982). The amount of cells is always given as dry weight (Holst et al. 1982).

Immobilization

The cells were immobilized in calcium alginate gel as described elsewhere (Adlercreutz and Mattiasson, in press). Preparations with varying particle sizes were obtained by extruding the cell-alginate slurry through hypodermic needles with varying diameters during immobilization. The diameters of the gel beads were measured with a microscope after storage overnight in a 0.1 M succinate buffer (pH 5.0) containing 10 mM CaCl_2 . The cell density of a certain preparation was calculated as the amount of cells immobilized (dry weight) divided by the volume of the beads. Immobilization of cells for determination of operational stability was carried out under sterile conditions.

Experimental set up for kinetic measurements.

All the short-term experiments were carried out in a 115 ml reactor containing a polarographic oxygen electrode (Radiometer). The reactor was placed in a thermostated water bath and the solution was stirred magnetically. The magnet was fixed on an axis of stainless steel which passed through the top of the reactor through a hypodermic needle. In

this way the solution in the reactor could be stirred without attrition of the alginate beads which takes place if ordinary magnetic stirring is used.

The buffer used in all experiments (except when pH was varied) was a 0.1 M succinate buffer (pH 5.0) containing 10 mM CaCl_2 . The buffer was poured into the reactor where it was thermostated and saturated with air or pure oxygen. The cells were then added as either free cells suspended in 0.9% NaCl-solution or immobilized cells and the lid, a rubber stopper, was put on.

The signal from the oxygen electrode was monitored and recorded. When the signal had stabilized, the substrate (glycerol) was added through a hypodermic needle. Usually glycerol was added to a final concentration of 0.25 M. The signal from the oxygen electrode was continuously recorded and the reaction rate could be calculated from the slope of the curve.

In most experiments, just the initial reaction rate was determined. However, as the oxygen concentration in the reactor decreased continuously, the reaction rate at different oxygen concentrations could be measured in the same experiment.

Bubble column reactor experiments

About 75 g of alginate beads, corresponding to 5 g dry wt/L reactor volume, were placed in a bubble column reactor with a working volume of 500 mL. The reactor had an inner diameter of 5.5 cm and was 30 cm high. The supply of oxygen was assured by forcing air through a sintered glass filter in the bottom of the reactor in order to form small gas bubbles. The air flow rate was 1.0 vvm. To ensure that the oxygen concentration was sufficient to fulfill the microorganism's requirements, oxygen was measured with a galvanic oxygen electrode (Johnson et al 1964) connected to a recorder. The dissolved oxygen level was never below 90% of air saturation.

In order to obtain good mixing properties, the reactor was slightly tapered at the bottom. The reactor was also double walled to facilitate temperature control by circulating water from a water bath. The temperature was maintained at 30°C. The medium consisted of glycerol (0.25 M) in succinate buffer (0.1 M, pH 5.0). Calcium chloride (10 mM) was added in order to stabilize the gel. Medium was fed with a membrane pump (Braun Melsungen FE 211). The volume was kept constant by suction from the surface with a peristaltic pump. The experiments were carried out under sterile conditions.

Solubilization of alginate gel.

In order to check the activity of the cells after immobilization, alginate beads were dissolved in 0.1 M citrate buffer (pH 5.0). The buffer was stirred magnetically and dissolution was complete in approximately 30 min. The activity of the cells was then determined as above.

Analysis of dihydroxyacetone

DHA was assayed with a modification of the DNS-method of Miller et al. (1960). In this method DHA is oxidized by dinitrosalicylic acid and a coloured compound is formed. One ml of the sample was mixed with 1 ml of the DNS-reagent. The absorbance was measured at 640 nm by Miller et al., although the absorbance maximum is at much shorter wavelength. The sensitivity of the method was increased in this study by measuring the absorbance at 575 nm. This increased the absorbance by a factor of 3.5. At still shorter wavelengths (470-520 nm) the method is unreliable (Hostettler et al. 1951).

Oxygen analysis by Winkler titration.

The method used in this study was a modification of the method in ASTM Standards (American Society for Testing and Materials 1980). The

modification was necessary because of the high buffering capacity of the samples. The reagents were the same as in the standard method but the amounts had to be increased. The sample was poured into a bottle with a volume of 116.7 ml. Then 1.4 ml of alkaline potassium iodide solution (150 g/l KI, 700 g/l KOH) and 0.7 ml of manganese sulphate solution (364 g/l $\text{MnSO}_4 \cdot \text{H}_2\text{O}$) were added and the stopper was sealed tightly. After completion of the reaction, 2.1 ml of H_2SO_4 (3:1) was added. The bottle was stoppered and shaken until the precipitate was dissolved. The liberated iodine was titrated with a sodium thiosulphate solution.

Results and Discussion

Cell immobilization

The immobilization resulted in spherical gel beads. No cells could be detected in the calcium chloride solution after immobilization as determined by measuring the glycerol oxidizing capacity. In a typical batch 76.8g of cell-alginate slurry gave 37.9g of wet catalyst, the shrinkage thus being 51%. The particle diameter was quite uniform, a typical result being 2.20 ± 0.06 mm (mean $\pm$ standard deviation).

In order to check if the immobilization procedure harmed the cells, the beads were dissolved in citrate buffer after immobilization and their activity was determined. It was found that their activity was the same as that of cells that had not been immobilized. Additions of citrate and sodium alginate to the medium had no effect on the activity of the cells.

Kinetic experiments.

In the kinetic experiments, the rate of glycerol oxidation to DHA by G. oxydans cells was measured under different conditions. A buffer solution was poured into a 115 ml reactor where it was thermostated and saturated with oxygen or air. The cells were suspended in the solution.

Then glycerol was added and the reaction started. The oxygen concentration was continuously measured with a polarographic oxygen electrode. It was found that the rate of oxygen transfer was the rate limiting factor. Therefore, straight graphs were obtained. When immobilized cells were used, bent graphs were obtained because oxygen transfer was the rate limiting factor.

From the slope of the curves, the reaction rate could be calculated. In the following experiments, the reaction rate was measured as a function of different parameters.

Reaction rates were recalculated by converting the electrode measurement from partial pressure of oxygen to a concentration value after standardizing the oxygen concentration with the Winkler-method.

The oxygen concentration in the succinate buffer was determined by Winkler titration. The oxygen solubility in 0.1 M sodium succinate buffer (pH 5.0) containing 10 mM CaCl_2 and 0.25 M glycerol, as used in the kinetic experiments, was 91.5% of that in pure water. The determinations were done with air saturated liquids at 30°C. As the concentration of oxygen in pure water saturated with air (1 atm at 30°C) is 7.57 ppm (Montgomery et al. 1964), the concentration in the buffer solution under these conditions was 0.216 mM. If the buffer solution had been saturated with pure oxygen, the solubility would be 1.034 mM.

Influence of temperature

The temperature dependence of the reaction was studied. The optimal temperature for the initial reaction rate was 40°C (Fig. 2). But when choosing a suitable temperature for long term operation, the stability of the cells must also be considered. In figure 2, the initial reaction rates are given. The remaining activity (measured at 25°C) after 24 h of operation at different temperatures is shown in figure 3. The activity of free cells decreased rapidly with increasing temperature. The immobilized cells were more stable. After operation at 25°C, the activity was a little higher than before treatment. Apparently some activation of the cells or some limited cell growth had taken place. Operation at temperatures above 35°C decreased the activity of the immobilized cells considerably. For the rest of the experiments, a temperature of 30°C was chosen.

The disappearance of oxygen was measured in all of these measurements. The most important parameter was however, the production of DHA. In order to check if the disappearance of oxygen was accompanied by a corresponding increase in the concentration of DHA, the temperature dependence experiments were repeated and the concentration of DHA was measured. DHA concentration was determined by a modified DNS-method (see Materials and Methods). The results showed, with respect to the stoichiometry, that the reaction rate measured as the increase of DHA concentration was the same as the rate measured as the decrease in oxygen concentration.

Influence of pH

It was found that a pH of 5 was optimal for the production of DHA by both free and immobilized G. oxydans cells (Fig. 4). The pH-optimum curve in this figure was flat especially when immobilized cells were used. These observations agree well with previous studies of this organism (Holst et al. 1982).

Influence of glycerol concentration

The activity of the cells as a function of the glycerol concentration is presented in figure 5. The curves are sigmoidal both for free and immobilized cells, indicating that Michaelis-Menten kinetics are not applicable. The glycerol concentration required to get half the maximal reaction rate was about 5 mM both for free and immobilized cells. In all the remaining experiments, a glycerol concentration of 0.25 M was used unless otherwise stated. At this concentration, glycerol is not a limiting factor.

The influence of oxygen concentration, cell density and particle size.

The activity of free cells is independent of the oxygen concentration in a wide concentration range. However, the activity of immobilized cells varied with the oxygen concentration (Fig 6). Thus, if the production medium is saturated with pure oxygen instead of air the production rate can be greatly increased.

In order to investigate how the particle size and the cell density of the gel influenced the productivity, immobilized preparations with different cell loadings and particle sizes were made. The activity of the cells decreased with increasing particle size, as expected (Fig. 7). Even with the smallest particle size, the activity was far below that of free cells. In the other set of preparations, the particle size was kept constant and the cell density was varied. As expected, the activity decreased with increasing cell density (Fig. 8).

When immobilized aerobic cells are used, oxygen supply is often a limiting factor (e.g. Tramper et al. 1983). As the transfer of oxygen from the bulk liquid phase to the cells in the matrix is a slow process. The oxygen concentration in the centre of the beads will be low, if not zero, so that only the cells at the surface of the beads will be supplied with enough oxygen to carry out the oxidation reaction at a normal rate. The mean activity of cells in an immobilized preparation will therefore be considerably lower than that of free cells.

The activity of immobilized aerobic cells is dependent on several parameters. The three most important of these are the cells density, the particle size and the bulk concentration of oxygen. If a low cell density, a small particle size and a high bulk concentration of oxygen is used, the activity of free cells can be approached.

A thorough discussion of the importance of the different parameters accompanied by a computer simulation will be published shortly (Adlercreutz, in prep).

Product inhibition

The oxidation of glycerol to DHA is not inhibited to any great extent by the product DHA. Only at a very low glycerol concentration and a very high DHA concentration was a small inhibitory effect noticed (Table 1).

Operational stability

The operational stability of the immobilized preparation was studied in a continuously operated bubble column reactor. The reactor was operated for 12 days with a dilution rate of 0.15 h^{-1} giving a residence time of 6.67 h.

The medium used consisted of glycerol (0.25 M) in succinate buffer (pH 5.0, 0.1M) supplemented with CaCl_2 (0.01 M).

Under these conditions, the half-life of the preparation was found to be 5 days (Fig. 9).

The mechanical stability of the beads was good. No turbidity, measured as optical density at 620 nm, could be detected in the outlet. A slight swelling of the beads was noted. The diameter increased from 2.1 mm to 2.5 mm.

Experiments to investigate the influence of dilution rate (residence time) on the conversion degree were difficult to carry out due to the rather low operational stability. When long residence times were used and a throughput of several reactor volumes was allowed, the decrease in activity made measurements meaningless.

Storage stability

Free and immobilized cells were stored in succinate buffer at 4°C for periods up to 68 days. Under these conditions the cells were quite stable. A slight decrease in the activity of free cells was observed while no decrease at all could be observed with the immobilized cells (Fig. 10). Storage in a salt solution (0.15 M NaCl, 10 mM CaCl₂) at 4°C gave similar results (data not shown).

Concluding Remarks

The present investigation was focused on immobilized resting cells. The reaction studied in this system is well suited for use in the study of a conversion by an immobilized cell preparation. This is because the conversion is a one-step process, the substrate is a small molecule and, finally, that the product is not charged. These facts reduce the environmental influence on the cells in the immobilized state.

A limiting factor, however, is the low solubility of the second substrate, oxygen.

From the studies on the effects of pH and temperature on the immobilized preparations, it is seen in figures 2 and 4, that the profiles are broader than the corresponding curves for the free cells. This may be ascribed to the fact that due to diffusion limitations, an excess of catalytic activity was present under optimal conditions as only the outer layer of the immobilized cells were exposed to the substrate. When the conditions were changed and less substrate was converted by the initially active cells substrate became available to cells placed deeper in the matrix which in turn reduced the observed effect of the changed conditions. In the figures giving the pH-activity profiles as well as that showing the temperature dependence profiles, are seen one curve in each graph obtained, using a preparation of immobilized cells with a reduced cell density. With reduced cell densities the diffusion restrictions found with immobilized cells were less pronounced and thus the appearance of the pH-activity and temperature profiles were closer to those of free cells.

When characterizing a preparation it is easier to use resting cells in comparison to a situation when the cells are growing, since in the

latter case leakage of cells from the support to the surrounding medium may take place. This leads to a situation with a population of both immobilized cells and freely suspended cells. Determination of kinetic parameters valid for immobilized cells would under such circumstances involve severe experimental problems. However, for actively growing immobilized cells far better operational stability of the immobilized preparation has been demonstrated by e.g. Seiskari et al (1984).

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References:

Adlercreutz P, Holst O, Mattiasson B (1982)

Oxygen supply to immobilized cells: 2. Studies on a coimmobilized algae-bacteria preparation with in situ oxygen generation. *Enz Microb Technol* 4:395-400

Adlercreutz P, Mattiasson B (1982)

Oxygen supply to immobilized cells: 3. Oxygen supply by hemoglobin or emulsions of perfluorochemicals. *Eur J Appl Microbiol Biotechnol* 16:165-170

Adlercreutz P, Mattiasson B (1984)

Oxygen supply to immobilized cells: 4. Use of p-benzoquinone as an oxygen substitute. *Appl Microbiol Biotechnol* 20: 296-302

American Society for Testing and Materials (1980) Annual book of ASTM standards, Part 31, D 888, method E, p 538-539

European Federation of Biotechnology (1983) Guidelines for the characterization of immobilized biocatalysts. *Enz Microbiol Technol* 4:395-400

Green SR (1967) Dihydroxyacetone. In: *Microbial Technology* (Peppler H J ed) p 360, Reinhold Publ Corp, New York

Holst O, Enfors SO, Mattiasson B (1982)

Oxygenation of immobilized cells using hydrogen peroxide; A model study of Gluconobacter oxydans converting glycerol to dihydroxyacetone. *Eur J Appl Microbiol Biotechnol* 14:64-68

Hostettler F, Borel E, Deuel H (1951)

Über die Reduktion der 3,5-Dinitrosalicylsäure durch Zucker. *Helv Chim Acta* 34:2132-2139

Johnson MJ, Borkowski J and Engblom C (1964)

Steam Sterilizable Probes for Dissolved Oxygen Measurement. *Biotechnol Bioeng* 6:457-468.

Makhotkina TA, Pomortseva, NV, Lomova IE, Nikolaev, PI (1981)

Glycerol transformation into dihydroxyacetone by polyacrylamide gel immobilized cells of Gluconobacter oxydans. *Prikl Biokhim Mikrobiol* 17:102-106

Miller GL, Blum R, Glennon WE, Burton AL (1960)

Measurements of carboxymethylcellulase activity. *Anal Biochem* 2:127-132

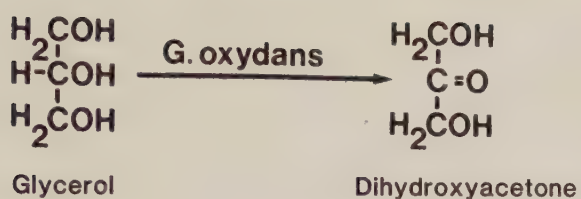
Montgomery HAC, Thom NS, Cockburn A (1964) Determination of dissolved oxygen by the Winkler method and the solubility of oxygen in pure water and seawater. *J Appl Chem* 14:280-296

Nabe K, Izuo N, Yamada S, Chibata I (1979)

Conversion of glycerol to dihydroxyacetone by immobilized whole cells of Acetobacter xylinum. *Appl Environ Microb* 38:1056-1060

Tramper J, Luyben KChAM, van den Tweel WJJ (1983)

Kinetic aspects of glucose oxidation by Gluconobacter oxydans cells immobilized in Calcium alginate. *Eur J Appl Microbiol Biotechnol* 17:13-18



Reaction scheme

Table 1

Glycerol (mM)	DHA (mM)	Activity (mmol O ₂ /h·g)
250	0	6.48
250	250	6.42
250	1000	6.06
10	0	5.94
10	250	5.35
10	1000	4.72

Table 1. Product inhibition of glycerol oxidation to DHA by immobilized G. oxydans cells. In each experiment, 25 mg of cells were used. Cell density was 29 g/l and the particle diameter was 2.3 mm. The temperature was 30°C. All substrate solutions were saturated with oxygen.

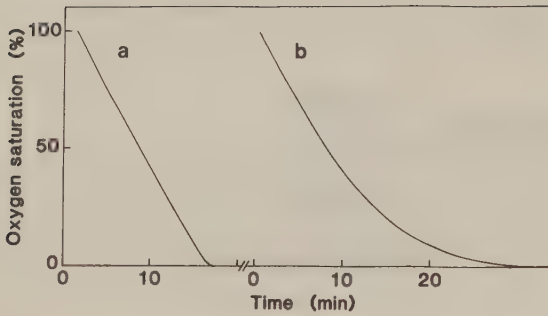


Figure 1. Oxygen concentration as a function of time as recorded in the kinetic measurements. The oxygen concentration was continuously measured with a polarographic oxygen electrode. The diagram shows one experiment with free cells (a) and one with immobilized cells (b).

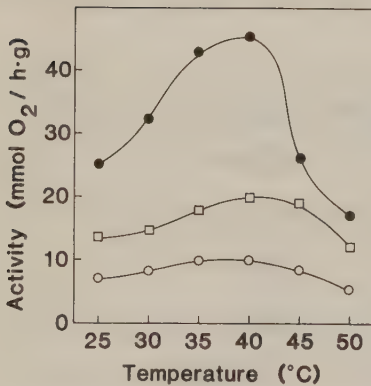


Figure 2. Temperature dependence of the oxidation of glycerol to DHA by *G. oxydans* cells. The glycerol concentration was 0.25 M. The substrate solution was oxygen saturated. Experiments were performed with either 10 mg of free cells or 25 mg of immobilized cells. The immobilized preparations had the cell densities of 9.6 g/L (□) and 29 g/L (○). The particle diameters were 2.1 mm and 2.3 mm, respectively. The activities for free (●) and immobilized (□, ○) cells are shown in the figure.

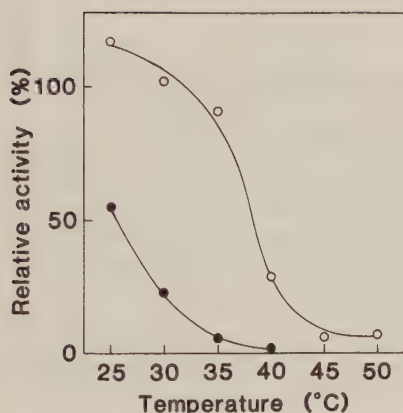


Figure 3. Temperature stability of *G. oxydans* cells while converting glycerol to DHA. In each experiment, 10 mg of free cells or 25 mg of immobilized cells (cell density: 29 g/l, particle diameter: 2.3 mm) were used. These cells were put in shaker flasks containing 150 ml of 0.50 M glycerol in succinate buffer. The flasks were shaken at different temperatures for 24 hours. Then the immobilized cells were recovered by filtration and the free cells by centrifugation (17000 g, 25 min). The remaining activity of the cells was then measured at 25°C using oxygen saturated media containing 0.25 M of glycerol. The activity of untreated cells was set at 100%. Data for free (●) and immobilized (○) cells are shown in the figure.

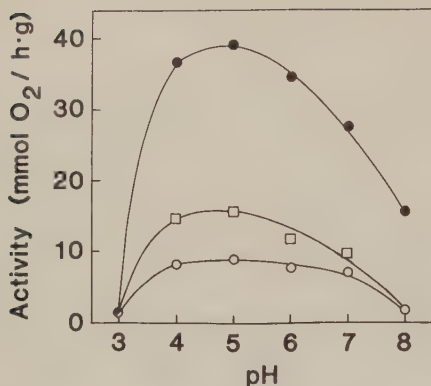


Figure 4. The pH-dependence of the glycerol oxidation to DHA by *G. oxydans* cells. The substrate solutions contained 0.25 M of glycerol in the following buffers; at pH 3, 0.1 M fumarate; at pH 4-6, 0.1 M succinate and at pH 7-8, 0.1 M tris. All buffers were supplemented with 10 mM CaCl_2 . The substrate solutions were saturated with oxygen and their temperatures were 30°C. Experiments with free cells used 10 mg of cells while experiments with immobilized cells were done with 25 mg of cells. The immobilized preparations had cell densities of 9.6 g/L (□) and 29 g/L (○). The particle diameters were 2.1 mm and 2.3 mm, respectively. The activities for free (●) and immobilized (□,○) cells are shown in the diagram.

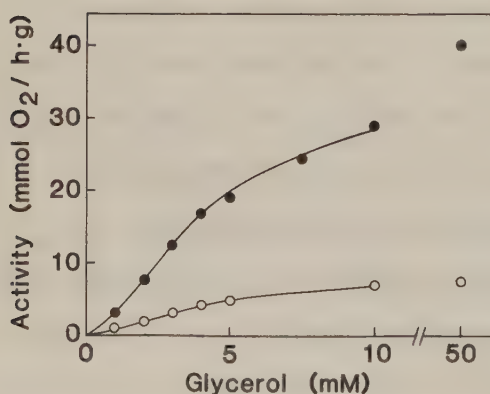


Figure 5. Productivity of *G. oxydans* cells as a function of the glycerol concentration. The temperature was 30°C. The substrate solutions were saturated with oxygen. Either 10 mg of free cells (●) or 25 mg of immobilized cells (○) were used in each experiment. Cell density was 29 g/l and the particle diameter was 2.3 mm.

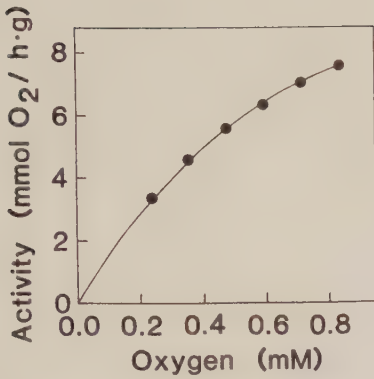


Figure 6. Productivity of immobilized *G. oxydans* cells as a function of the oxygen concentration in the bulk liquid phase. The amount of immobilized cells used was 25 mg. The particle diameter was 2.3 mm and the cell density was 29 g/l. The glycerol concentration was 0.25 M and the temperature was 30°C.

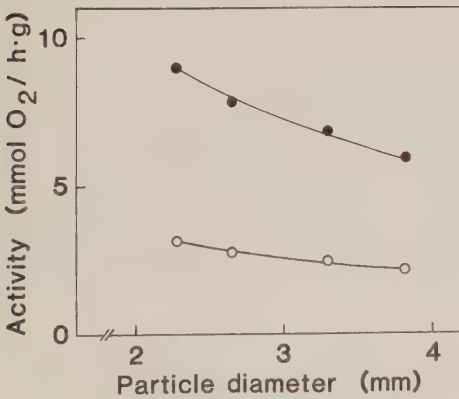


Figure 7. Productivity of immobilized *G. oxydans* cells as a function of the particle diameter. The glycerol concentration was 0.25 M and the temperature was 30°C. In each experiment, 25 mg of cells were used. The cell density was 25-29 g/l. For each particle diameter' the productivities at oxygen concentrations corresponding to saturation with air (○) or oxygen (●) are given.

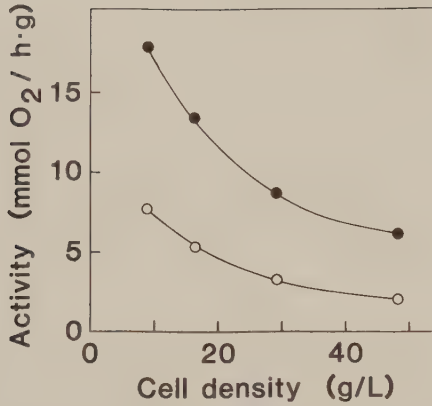


Figure 8. Productivity of immobilized G. oxydans cells as a function of the cell density in the alginate gel. The glycerol concentration was 0.25 M and the temperature was 30°C. In each experiment, 25 mg of cells were used. The particle diameters were 2.2 - 2.4 mm. For each cell density, the productivity at an oxygen concentration corresponding to saturation with air (○) or oxygen (●) is shown.

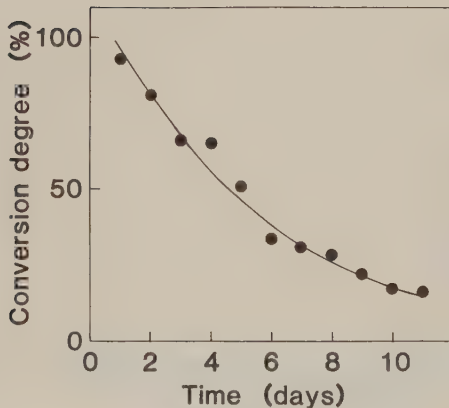


Figure 9. Operational stability, expressed as conversion degree as a function of time, of G. oxydans immobilized in calcium alginate. The residence time was 6.67 h ($D = 0.15 \text{ h}^{-1}$). The glycerol concentration was 0.25 M and temperature was 30°C.

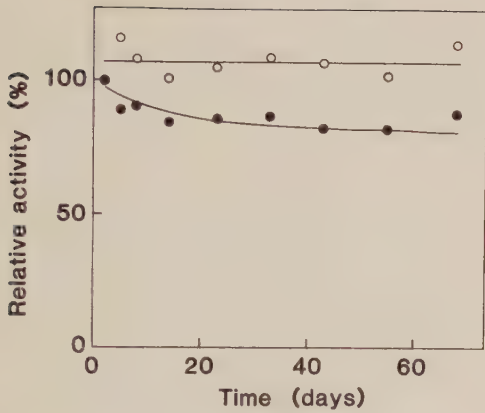


Figure 10. Storage stability of *G. oxydans* cells. Each aliquot of cells consisted of 10 mg of free cells or 25 mg of immobilized cells. The cell density was 29 g/l, and the particle diameter was 2.3 mm. The cells were stored in 0.1 M succinate buffer (pH 5.0) containing 10 mM of CaCl_2 at 4°C for different periods of time. The activity was determined at 30°C with the glycerol concentration of 0.25 M. The activity, for free cells (●) and for immobilized cells (○), at an oxygen concentration of 0.90 mM (87% oxygen saturation) is shown.

Oxygenation of Immobilized Cells Using Hydrogen-Peroxide; A Model Study of *Gluconobacter oxydans* Converting Glycerol to Dihydroxyacetone

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Summary. The possibility of using hydrogen-peroxide as an oxygen source in immobilized whole cell systems was investigated.

The conversion of glycerol to dihydroxyacetone performed by Ca-alginate immobilized *Gluconobacter oxydans* was used as a model system. The influence of basic parameters, such as pH, temperature and concentration of hydrogen-peroxide, on the conversion are reported. Continuous production using a packed bed could be substantially improved by using hydrogen-peroxide as a source of oxygen. The productivity was increased almost twenty-fold in the reactor. However, hydrogen-peroxide also caused deactivation of the oxidizing of the cells.

capacity

Introduction

Most of the work, so far reported, concerning immobilized whole cells deals with processes that require small or no amounts of oxygen e.g. anaerobic processes such as production of ethanol (Kierstan and Bucke 1977, Sitton and Gaddy 1980; Wada et al. 1980), production of organic solvents (Häggström and Molin 1980) and denitrification of water (Nilsson et al. 1980). However, some authors have investigated the use of immobilized whole cells for aerobic processes such as the use of acetic acid bacteria for bioconversion (Schnarr et al. 1977; Nabe et al. 1979; Makhotkina et al. 1980; Tramper et al. 1981). These authors report on the oxygen diffusion limitations in the systems, which is one of the main drawbacks of immobilized (entrapped) whole cells in aerobic processes.

The gas-liquid oxygen transfer rate (OTR) in microbial processes is described by the equation $OTR = K_1 a (C^* - C)$

where K_1 denotes the overall oxygen transfer coefficient (l/h). C^* is the saturation dissolved oxygen concentration (mg/l) in the medium and C is the actual dissolved oxygen concentration (mg/l) in the medium. This equation suggests that OTR can be increased by increasing a or C^* e.g. by I) increased agitation II) increased partial-pressure of oxygen and III) increased gas-flow rate. Increased agitation is almost impossible in systems with immobilized whole cells due to the mechanical properties of the gel. High gas-flow rates will, for the same reason, be difficult to use. With these restrictions OTR could only be changed within narrow limits due to the low solubility of oxygen in media.

In this work the possibility of using hydrogen-peroxide to improve the oxygen supply in immobilized whole cell systems is investigated. The conversion of glycerol to dihydroxyacetone (DHA) by *Gluconobacter oxydans* ATCC 621 immobilized in Ca-alginate was chosen as a model (Fig. 1). The conversion is strictly aerobic and organisms belonging to the genus *Gluconobacter* have high catalase activity. The catalytic decomposition of hydrogen-peroxide thus takes place inside the beads thereby reducing the transport problems connected with oxygen transfer.

Materials and Methods

Chemicals. Sodium alginate and Catalase (EC 1.11.1.6, from Bovine liver, 3,000 U/ μ l) was obtained from Sigma Chemicals Co., USA. Hydrogen-peroxide (30%, Perhydrol) was purchased from Merck, Darmstadt, FRG. All other chemicals were of analytical grade and obtained from commercial sources.

Cultivation of Microorganisms. *Gluconobacter oxydans* (ATCC 621) was maintained on agar slants of the following composition (in g/l): glycerol 20.0; agar 20.0; yeast extract 5.0; peptone 3.0; NH_4Cl 0.8; $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ 2.7; KH_2PO_4 11.6; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.2; trace elements (in mg/l): $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ 8.35; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.33; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 0.09; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ 0.09; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 0.08; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ 0.08.

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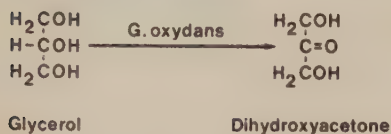


Fig. 1. Reaction scheme

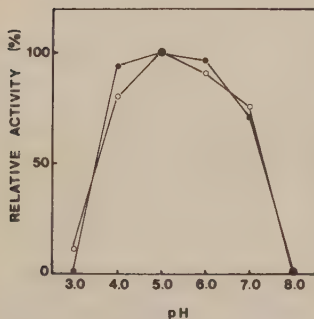


Fig. 2. The effect of pH on the conversion of glycerol to dihydroxyacetone performed by Ca-alginate immobilized *G. oxydans* using air or hydrogen-peroxide as the source of oxygen. The different pH values were obtained using buffers as follows: pH-interval 3.0–6.0, fumarate; 6.0–7.0, maleate; 7.0–8.0 Tris HCl. The medium contained buffer as above, glycerol 0.5 M and CaCl_2 5mM. The hydrogen-peroxide concentration used was 100 mM. When air was used as the oxygen source 10 g alginate beads were vigorously shaken in baffled Erlenmeyer flasks with 100 ml medium at 28 °C for 2 h. When hydrogen-peroxide was used as the oxygen source 5 g alginate beads were gently shaken in unbaffled bottles with 200 ml medium at 28 °C for 1 h. Maximum activity, expressed as gram DHA produced/gram cells and hour was set to 100% in each case. (—●—) air; (---○---) hydrogen-peroxide

The culture was reinoculated on agar slants every month.

Microorganisms from these agar slants were transferred to 150 ml medium in 2,000 ml baffled Erlenmeyer flasks and cultivated for 24 h at 28 °C in a rotary shaker. This was used as inoculum in the fermentor cultivations. In preparing the inoculum the same medium as above, except agar, was used. Batch cultivations were performed in a fermentor (Chemoform AB, Hägersten, Sweden) with a working volume 3 l. Temperature and pH were controlled at 28 °C and 6.0, respectively; 1 M NaOH was used to keep pH constant. Foaming was controlled by adding, when needed, the antifoam Adekanol LG-109 (Asahi Electro Chemical Co., Japan). The medium in the fermentor was inoculated with 5% (v/v) inoculum. In the batch cultivations medium of the following composition was used (in g/l): glycerol 50.0; yeast extract 5.0; peptone 3.0; NH_4Cl 0.8; $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ 0.6; KH_2PO_4 0.4; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.2 and trace elements as above.

Determination of Dry Weight. Cell dry weight was measured in samples of the fermentation broth. After centrifugation (17,600 g, +4 °C, 25 min) of a defined volume of cell suspension, the pellets obtained were washed with 0.9% NaCl and dried over-night at 105 °C. Biomass concentrations are expressed as dry weights unless otherwise stated. The cell yield was on average 2.4 g dry wt/l.

Harvesting. The organisms were harvested in the early stationary phase. Cells for immobilization were collected by centrifugation (17,600 g, +4 °C, 25 min) and washed once with 0.9% NaCl.

Immobilization in Ca-alginate. *G. oxydans* cells were immobilized as follows: 1 g (wet wt.) of cells were mixed thoroughly with 9 g of a 2% (w/v) sodium-alginate solution. The mixture was pumped continuously through a hypodermic needle (1.0 mm I.D.) and dropped into a solution of 0.1 M CaCl_2 to form beads. The beads were stabilized in a fresh solution of 0.1 M CaCl_2 at room temperature 1–2 h. After washing briefly with water the beads were stored in a moist condition at +4 °C before use. About 5 g beads of an average diameter of 3 mm were obtained. The cell content in the beads was appr. 30 mg dry wt./g wet gel.

Identification and Quantification of Dihydroxyacetone. Identification of dihydroxyacetone was made on Kieselgel 60, 10 × 10 cm, high performance thin layer chromatography (HPTLC) plates (Merck, Darmstadt, FRG). The plates were eluted using ethyl acetate:acetone:water (40:50:10) as solvent. DHA and glycerol were revealed using KMnO_4 in sodium-hydroxide according to Stahl (1969). DHA yielded a brown spot and glycerol a white spot on a light brown background. DHA in the samples was identified by direct comparison with pure DHA (Merck, Darmstadt, FRG). Dihydroxyacetone was assayed as a reducing substance using the DNS-method according to Miller et al. (1960). Since hydrogen-peroxide interfered with the analysis, residual hydrogen-peroxide was removed by the addition of a small amount of catalase (5 µl/0.5 ml sample) prior to the addition of the reagent.

Assay of the Effect of Different Hydrogen-peroxide Concentrations. Alginate beads (3.0 g) containing immobilized *G. oxydans* were incubated in 300 ml medium (0.5 M glycerol and 5 mM CaCl_2 in 0.1 M succinate buffer, pH 5.0, and various concentrations of hydrogen-peroxide) at 25 °C.

To maintain the concentration of hydrogen-peroxide at the predetermined value the beads were withdrawn, washed and supplied with fresh medium every second hour. Samples were taken every hour and the concentration of DHA was determined. The concentrations of hydrogen-peroxide used were 0, 1, 2, 5, 10, 15, 25, 50 and 100 mM.

Results and Discussion

The Effect of pH

The pH activity profiles for the conversion of glycerol to DHA using air or hydrogen-peroxide as the source of oxygen were examined in the pH-range 3.0–8.0 (Fig. 2). They were found to be highly similar with the pH-optima at 5.0. In the subsequent experiments this optimum pH was used. Essentially the same pH optimum has been reported for other immobilized acetic acid bacteria converting glycerol to DHA (Nabe et al. 1979).

The Effect of Temperature

The effect of temperature was investigated in the range of 20 to 50 °C using air or hydrogen-peroxide as oxygen

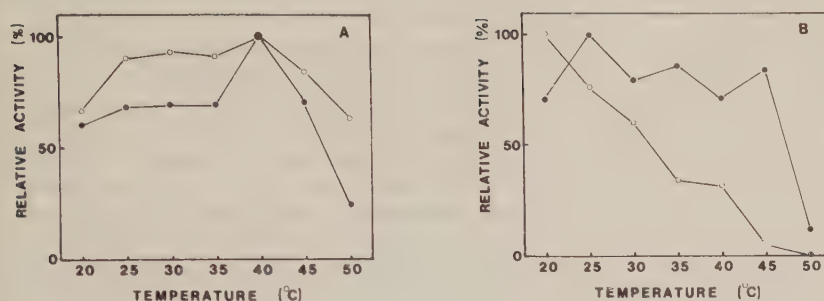


Fig. 3A and B. The effect of temperature on the conversion of glycerol to dihydroxyacetone performed by Ca-alginate immobilized *G. oxydans* using air or hydrogen peroxide as the source of oxygen. The medium contained 0.5 M glycerol and 5 mM CaCl_2 in succinate buffer (0.1 M; pH 5.0). The hydrogen-peroxide concentration used was 100 mM. When air was used as the source of oxygen 5 g alginate beads were vigorously shaken in baffled Erlenmeyer flasks with 100 ml medium at indicated temperatures for 2 h. When hydrogen-peroxide was used as the source of oxygen 5 g alginate beads were gently shaken in unbaffled bottles containing 200 ml medium at indicated temperatures for 1 h (Fig. 3A). Maximum activity, expressed as gram DHA produced/gram cells and hour, was set as 100% in each case. After treating the immobilized cells as described above the beads were collected and washed. To determine the remaining activity the beads were vigorously shaken in baffled Erlenmeyer flasks with 100 ml medium at 30 °C for 2 h using air as the source of oxygen (Fig. 3B). Maximum retained activity, expressed as gram produced DHA/gram cells and hour, was set as 100% in each case. (—●—) air; (—○—) hydrogen-peroxide

source (Fig. 3a and b). From Fig. 3a it can be seen that the temperature optimum for the initial conversion rate is similar and broad using either oxygen source. A rapid decrease in activity occurs when temperature rises above 45 °C. Similar results have been reported for the conversion of glycerol to DHA using *Acetobacter xylinum* immobilized in polyacrylamide (Nabe et al. 1979). The temperature stabilities, however, show differences when air or hydrogen-peroxide are used as oxygen sources (Fig. 3b). The activity, when air is used, shows good stability up to 45 °C when a sudden drop occurs. When hydrogen-peroxide is used the stability declines in the whole temperature range thus showing the best stability at low temperatures. This has been interpreted as being due to the deleterious effects of hydrogen-peroxide. The velocities of the harmful reactions in which hydrogen-peroxide participate probably increase with increased temperatures. To obtain high activity and high stability of the immobilized cell preparations 25 °C was used in the subsequent experiments.

The Effect of Hydrogen-peroxide

To investigate the effect of hydrogen-peroxide, alginate beads containing *Gluconobacter oxydans* were incubated in different concentrations of hydrogen-peroxide. Since hydrogen-peroxide is consumed during the reaction, the medium was withdrawn and replaced with fresh medium every second hour thus simulating batch processes with constant hydrogen-peroxide concentrations.

The main effect of hydrogen-peroxide was to drastically reduce the time needed for producing DHA. This is illustrated in Fig. 4, where the time needed for producing an arbitrarily chosen amount of DHA is plotted as a function of the concentration of hydrogen-peroxide.

Furthermore, hydrogen-peroxide affects the long-term stability of the preparation. A rapid decrease in activity when using high concentrations of hydrogen-peroxide is seen in Fig. 5, where the remaining activity after 10 h incubation compared to the initial activity is shown as a function of the hydrogen-peroxide concentrations used. This figure clearly illustrates the disadvantage of using high concentrations of hydrogen-peroxide. However, when 10 mM hydrogen-peroxide was used 100% of the activity remained after 10 h.

It should be stressed that the amount of living cells after incubation in high concentrations of hydrogen-peroxide were not investigated in detail. Cells that have been oxygenated with air for a long time might be able to proliferate while there are indications that cells incubated in 100 mM hydrogen-peroxide for 10 h are irreparably damaged. Since this affects the possibility of re-incubation, further investigations are needed.

Continuous Operation

Continuous production of DHA from glycerol using *G. oxydans* immobilized in Ca-alginate is shown in Fig. 6. During the first 20 h medium saturated with air was used to sup-

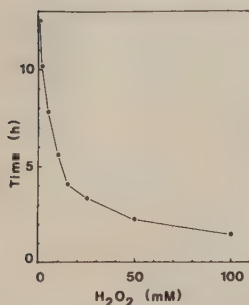


Fig. 4. Time for the production of an arbitrarily chosen amount (3.33 g DHA/g cells) of dihydroxyacetone by *G. oxydans* immobilized in Ca-alginate as function of the hydrogen-peroxide concentration.

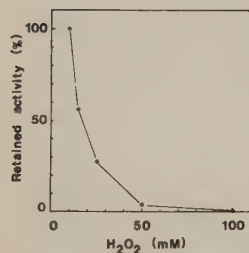


Fig. 5. Change in activity of immobilized *G. oxydans* converting glycerol to dihydroxyacetone using hydrogen-peroxide as the source of oxygen. Retained activity is expressed as the relationship between activity after 10 h and the initial activity. The activity is calculated as gram DHA/gram cells and hour.

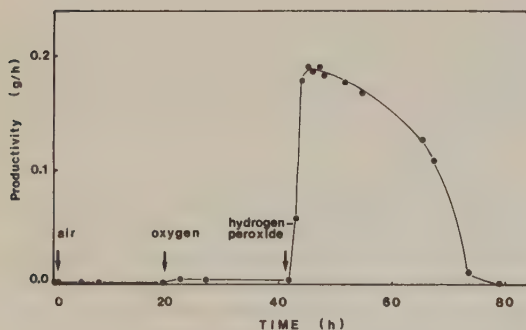


Fig. 6. Continuous production of dihydroxyacetone from glycerol using *G. oxydans* immobilized in Ca-alginate. Alginate beads containing 3.0% cells (dry wt) were packed in a column (bed volume: 40 ml). The column was fed with 0.1 M glycerol and 5.0 mM CaCl_2 in succinate buffer, pH 5.0, with a flow rate of 1.5 ml/min. During the first 20 h medium saturated with air was used. During the next 20 h the medium was saturated with oxygen and after a total of 40 h the medium was supplemented with hydrogen-peroxide to give a final concentration of 25 mM.

ply the organisms with oxygen for the conversion. Since this apparently is not enough the medium was instead saturated with pure oxygen. This resulted in a minor improvement of the conversion. However, the product concentration in the outlet still remained low. To improve this further hydrogen-peroxide was added to the medium to give a final concentration of 25 mM. As seen in Fig. 6 this markedly improved the conversion of glycerol to DHA. The productivity was increased about twenty-fold compared to the productivity when pure oxygen was used. The relatively high concentration of hydrogen-peroxide in the inlet of the column causes a rapid drop in activity. The half-life of the gel in the column was approximately 15 h.

The problem of deactivation might be circumvented if a batch-reactor with controlled feeding of hydrogen-peroxide is used as suggested earlier (Holst et al. 1981). This method provides the possibility to maintain a low but sufficient concentration of hydrogen-peroxide in the entire reaction vessel thus minimizing the deleterious effect connected with the use of this oxygen source. This approach will probably also make it easier to restore the oxidizing activity since the destruction of the cells will proceed slower at lower concentrations of hydrogen-peroxide according to Fig. 5.

General Discussion

The main resistance to oxygen transfer in immobilized cell systems lies in the transport of oxygen inside the matrix. As systems with immobilized whole cells cannot be subjected to high agitation rates nor high gas-flow rates other approaches must be made. Different attempts, which are applicable to systems with these restrictions, have been made to improve oxygen transfer in microbial processes. Increased oxygen partial pressure has been applied to the conversion of glycerol to dihydroxyacetone by *Gluconobacter melanogenus* in batch culture (Flickinger and Perlman 1977). This method has also been used for the same conversion using polyacrylamide immobilized *G. oxydans* with good results (Makhotkina et al. 1981).

Organic solvents, in which oxygen has a higher solubility than in water, have been used in steroid conversions (Buckland et al. 1975). Another attempt concerns the use of hydrogen-peroxide as a source of oxygen. Schlegel (1977) used hydrogen-peroxide instead of air for producing biomass from *Alcaligenes eutrophus* and other organisms. The hydrogen-peroxide was decomposed to water and oxygen using extra catalase added to the fermentor thereby providing the organisms with enough oxygen to maintain growth. However, difficulties arose when high cell densities were reached (Ibrahim and Schlegel 1980). The reason for this was attributed to several different mechanisms. There are also indications that growing cells are more sensitive to hydrogen-peroxide than resting cells (Schlegel 1977). This would favour the use of hydrogen-

peroxide as the oxygen source for immobilized whole cells in processes where the product formation is not growth associated.

Although high concentrations of hydrogen-peroxide affects the long-term stability of the immobilized *Gluconobacter oxydans*, converting glycerol to dihydroxyacetone, the reason for the deactivation of the preparation is still obscure. Hydrogen-peroxide itself might be the deleterious agent. Furthermore, very high local oxygen concentrations might occur in regions with high catalase activity and thus the formation of radicals such as the superoxide anion and the hydroxyl radical, which occur in all oxygen consuming reactions (Halliwell 1974, 1978), must be taken into account. Leakage of nucleotides from the cells has been suggested as a reason for loss in activity when transforming glycerol to dihydroxyacetone with polyacrylamide immobilized *Acetobacter xylinum* (Nabe et al. 1979).

To improve and restore the oxidizing capability of the preparation the possibility of re-incubation, by feeding nutrients to the organisms, must be considered.

In conclusion, the present report demonstrates one possible way to improve the oxygen supply for immobilized aerobic cells using hydrogen-peroxide as the oxygen donor.

Further studies on improving the stability of the system as well as on alternative oxygenation processes are now in progress.

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References

- Buckland BC, Dunnill P, Lilly MD (1975) The enzymatic transformation of water-insoluble reactants in nonaqueous solvents. Conversion of cholesterol to cholest-4-ene-3-one by a *Nocardia* sp. *Biotechnol Bioeng* 17:815–826
- Flickinger MC, Perlman D (1977) Application of oxygen-enriched aeration in the conversion of glycerol to dihydroxyacetone by *Gluconobacter melanogenus* IFO 3293. *Appl Environ Microbiol* 33:706–712
- Halliwell B (1974) Superoxide dismutase, catalase and glutathione peroxidase: solutions to the problems of living with oxygen. *New Phytol* 73:1075–1086
- Halliwell B (1978) Biochemical mechanisms accounting for the toxic action of oxygen on living organisms: The key role of superoxide dismutase. *Cell Biol Int Rep* 2:113–128
- Holst O, Enfors S-O, Mattiasson B (1981) The effect of improved oxygenation on the production of dihydroxyacetone from glycerol by *Gluconobacter oxydans* immobilized in alginate beads. *Abstr Comm Sec Eur Cong Biotechnol Eastbourne*, p 156
- Häggström L, Molin N (1980) Calcium alginate immobilized cells of *Clostridium acetobutylicum* for solvent production. *Biotechnol Lett* 2:241–246
- Ibrahim M, Schlegel HG (1980) Oxygen supply to bacterial suspensions of high cell densities by hydrogen-peroxide. *Biotechnol Bioeng* 22:1877–1894
- Kierstan M, Bucke C (1977) The immobilization of microbial cells, subcellular organelles, and enzymes in calcium alginate gels. *Biotechnol Bioeng* 19:387–397
- Makhotkina TA, Pomortseva NV, Lomova IE, Nikolaev PI (1981) Glycerol transformations into dihydroxyacetone by polyacrylamide gel immobilized cells of *Gluconobacter oxydans*. *Prikl Biokhim Mikrobiol* 17:102–106
- Miller GL, Blum R, Glennon WE, Burton AL (1960) Measurements of carboxymethylcellulase activity. *Anal Biochem* 2:127–132
- Nabe K, Izuo N, Yamada S, Chibata I (1979) Conversion of glycerol to dihydroxyacetone by immobilized whole cells of *Acetobacter xylinum*. *Appl Environ Microb* 38:1056–1060
- Nilsson I, Ohlson S, Häggström L, Molin N, Mosbach K (1980) Denitrification of water using immobilized *Pseudomonas denitrificans* cells. *Eur J Appl Microbiol Biotechnol* 10:261–274
- Schlegel HG (1977) Aeration without air: oxygen supply by hydrogen-peroxide. *Biotechnol Bioeng* 19:413–424
- Schnarr WG, Szarek WA, Jones JKN (1977) Preparation and activity of immobilized *Acetobacter suboxydans* cells. *Appl Environ Microbiol* 33:732–734
- Sitton OC, Gaddy JL (1980) Ethanol production in an immobilized cell reactor. *Biotechnol Bioeng* 22:1735–1748
- Stahl E (1969) *Thin-layer Chromatography*. Springer, Berlin Heidelberg New York
- Tramper J, Van den Tweel WJJ (1981) Continuous production of gluconic acid by an immobilized *Gluconobacter oxydans* system. *Abstr Comm Sec Eur Cong Biotechnol Eastbourne*, p 161
- Wada M, Kato J, Chibata I (1980) Continuous production of ethanol using immobilized growing yeast cells. *Eur J Appl Microbiol Biotechnol* 10:275–287

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DETERMINATION OF HYDROGEN PEROXIDE FOR APPLICATION IN AEROBIC CELL SYSTEMS OXYGENATED VIA HYDROGEN PEROXIDE

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SUMMARY

An amperometric method is described for the determination of hydrogen peroxide in reaction media used for production of dihydroxyacetone from glycerol by immobilized bacteria. The H_2O_2 was oxidized at +1.2 V vs. SCE at a glassy carbon flow-through electrode after dilution in a flow injection analysis system. Unexpected losses of H_2O_2 response in cell-free media necessitated a study of the cause. To validate the measurements two other methods for H_2O_2 determinations were used in parallel, a catalytic calorimetric method and an enzymatic flow injection method based on the use of immobilized peroxidase to produce a coloured product which was monitored spectrophotometrically. The three methods gave the same results. It was concluded that the responses of the methods were correct and that the loss of H_2O_2 is real and due to reactions with dihydroxyacetone or some of its decomposition products. A reverse reaction was also observed, i.e., the production of H_2O_2 from oxygen and decomposition products of dihydroxyacetone in an originally peroxide-free solution.

A suitable supply of oxygen is of the utmost importance for the productivity of aerobic cell systems, and the demands on the supply system become especially high in fermentors with high cell densities [1]. The solubility of oxygen in aqueous solutions is low compared to the normal substrate concentrations and the oxygen activity at the reaction site may be lowered still more by a slow mass transfer in the solution or within the cells. Enzymes such as catalase, present within the cell or in the cell membrane, can produce oxygen in situ through decomposition of hydrogen peroxide. Thus, addition of hydrogen peroxide to the medium is potentially a more efficient method of oxygenation than gas sparging. High concentrations of hydrogen peroxide may damage the cells, especially during the growth phase [2, 3], or may decrease the reaction rate by inhibition of enzymes. Increased productivity with hydrogen peroxide as the oxygen source has been demonstrated for both immobilized enzymes [4] and for resting immobilized cells [5].

Gluconobacter oxydans immobilized in beads of calcium alginate can be used for the production of dihydroxyacetone from glycerol [5]. The immobilized cells are alive but do not multiply during production of dihydroxyacetone and they are therefore more tolerant towards hydrogen peroxide than cells in the growth phase. The system is thus well suited for a methodical study of oxygenation through additions of hydrogen peroxide. A proper control of the peroxide concentration is nevertheless essential for high productivity without undue cell damage or inhibition.

This work is concerned with the development of methods for monitoring the hydrogen peroxide in the reactor with an electrochemical method very similar to that which has been used in earlier work on hydrogen peroxide determinations in pickling baths [6]. Because of an unexpected time dependence, it was compared to two other methods, each based on a different principle, in order to achieve mutual validation and assessment.

EXPERIMENTAL

Amperometric flow injection analysis

A 5- μ l sample was introduced into an unsegmented deaerated (vacuum) carrier stream of 0.2 M sodium acetate, pH 5.0, using a pneumatic injector. The sample was diluted 100 times in a dispersion coil and oxidized at a glassy carbon electrode, kept at +1.2 V vs. SCE by potentiostatic control. The electrode reaction is oxidation of hydrogen peroxide to oxygen and hydrogen ions, and the current is proportional to the concentration over a wide range. A new electrode is pretreated by keeping it at +1.2 V in a 10 mM peroxide solution until a stable value is obtained (some hours). After that, the electrode remains stable indefinitely if the potentiostat remains active. Further details of the method are available in an earlier paper [6].

Enzymatic spectrophotometric method

The flow injection system is outlined in Fig. 1.

A 25- μ l sample was introduced into a stream consisting of deaerated 0.1 M sodium phosphate, pH 6.0, by means of a pneumatically activated loop injector (Cheminert SVA 8031). Hydrogen peroxide from the sample oxidizes the immobilized peroxidase in the enzyme reactor and the peroxidase is then reduced during an oxidative coupling of 4-aminoantipyrine to 2,4-dichlorophenolsulphonate (DCPS). The coloured product is monitored at 514 nm in a 10- μ l flow cell (LKB model 2151). The flow rates were 0.70 and 0.35 ml min⁻¹ in the buffer and reagent lines, respectively.

Horse-radish peroxidase (EC 1.11.1.7, Sigma Type VI, Cat. No. 6140) was azo-immobilized [7, 8] onto porous glass (CPG-10, pore diameter 33 nm, 120–200 mesh; Electro Nucleonics, Fairfield, NJ). The enzyme-treated glass was packed into a reactor consisting of teflon tubing (1.0 mm i.d., 28 mm long, volume 22 μ l) connected to the detector with 0.3-mm i.d. teflon tubing. Mixing between reagent (10 mM DCPS, 1 mM 4-aminoantipyrine in

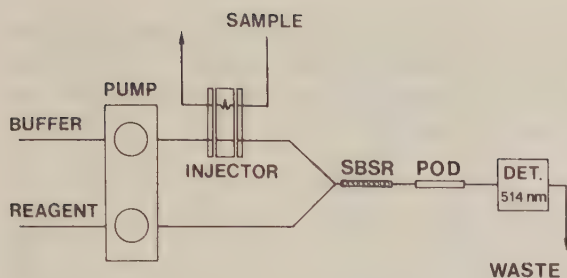


Fig. 1. Flow injection system for enzymatic determination of hydrogen peroxide. POD, peroxidase reactor; SBSR, single bead-string reactor.

0.1 M phosphate buffer, pH 6.0) and sample took place in a bead-string reactor [9] (i.d. 0.8 mm, length 100 mm, 0.5-mm diameter glass beads) which was inserted between the confluence point and the enzyme reactor. The samples were manually diluted 100 times before injection, to reduce the influence of substrate and products on the enzymatic reaction. The diluted samples were drawn into the sample loop by a separate peristaltic pump. The flow injection method was developed earlier at this laboratory for the determination of dissolved oxygen using other chromogens [10]. A thorough investigation of the oxidative coupling is under way.

Flow-through calorimetry

A 0.3-ml sample was introduced manually by a 4-way valve (Rheodyne) into a deaerated buffer stream consisting of 50 mM sodium succinate and 0.54 M glycerol, pH 5.0. Hydrogen peroxide from the sample was decomposed catalytically on platinized carbon (0.22 g) in a teflon reactor (i.d. 7 mm, length 18 mm) provided with a thermistor for temperature measurements [11]. A peristaltic pump (Bifok, FIA 08) was used to deliver the carrier solution (1 ml min^{-1}).

The reactor packing material was made by soaking granules of acid-washed carbon in hexachloroplatinic acid and drying it in an oven at 105°C for 1–2 h. The chloroplatinate was reduced at 300°C in a hydrogen stream until no acid fumes could be detected at the outlet. The amount of platinum was calculated to be 0.25 g g^{-1} of carbon. The calorimetric method has been described in detail elsewhere [12].

Chemicals

Hydrogen peroxide was obtained from Merck (Perhydrol, 30%). Stock solutions were standardized by titration with permanganate which in turn had been standardized against oxalate. A 5.54 M stock solution of dihydroxyacetone (98% pure; Merck) was prepared well in advance to ensure complete monomerization [13]. Methylglyoxal (grade II, 40% aqueous solution; Sigma) and DL-glyceraldehyde (>97%; Fluka), hexachloroplatinic(IV) acid (10%, p.a.; Merck), activated carbon (granules, 0.5–1.0 mm; KEBO AB,

Sweden) and 4-amino-1,2-dihydro-1,5-dimethyl-2-phenyl-3H-pyrazol-3-one (4-aminoantipyrine; BDH Chemicals) were used as received. DCPS was prepared as described by Barham and Trinder [14]. All other chemicals were from commercial sources and of p.a. quality.

RESULTS AND DISCUSSION

Amperometric method

The calibration graphs for hydrogen peroxide were strictly linear from 10^{-4} to 10^{-1} M. Electrochemical oxidation of glycerol, dihydroxyacetone or the buffer components takes place to some extent (see Table 1). The resulting currents were so small, however, that the interferences should be negligible in most cases (<6% for samples taken under the normal process conditions of 1–10 mM H_2O_2 , 0–1.1 M dihydroxyacetone, 1.1–0 M glycerol, pH 5.0).

The effect of dihydroxyacetone on the electrochemical oxidation of hydrogen peroxide was investigated by cyclic voltammetry (Fig. 2). It can be seen that increasing amounts of dihydroxyacetone affect both the peak shape and the peak position. The actual concentration of dihydroxyacetone at the glassy carbon electrode will be at most 11 mM because of the dilution in the dispersion coil. Thus its effect on the response to hydrogen peroxide can be neglected. The same conclusion was found to hold for additions of glycerol.

Reactions of dihydroxyacetone

Dihydroxyacetone mutually isomerizes with DL-glyceraldehyde; both isomers can react in irreversible, acid-base catalyzed steps to form methylglyoxal, which in turn may react slowly to form, e.g., lactic acid [15, 16]. The isomerization is catalyzed by base, or possibly acid/base [17], and is

TABLE 1

Peak currents from the oxidation of sample components in the amperometric flow cell (The interference level is the H_2O_2 sample concentration which gives the same peak current as the compound)

Compound	Conc. (M)	Current (nA)	Interference level (mM H_2O_2)
Dihydroxyacetone	1.1	3.5	0.052
	0.5	1.8	0.027
	0.25	1.0	0.015
Succinate	0.05	0.32	0.004
Glycerol	1.1	0.49	0.007
	0.54	0.18	0.003
	0.27	0.13	0.002
Methylglyoxal	0.002	<0.05	<0.0007
Glyceraldehyde	0.011	0.43	0.004

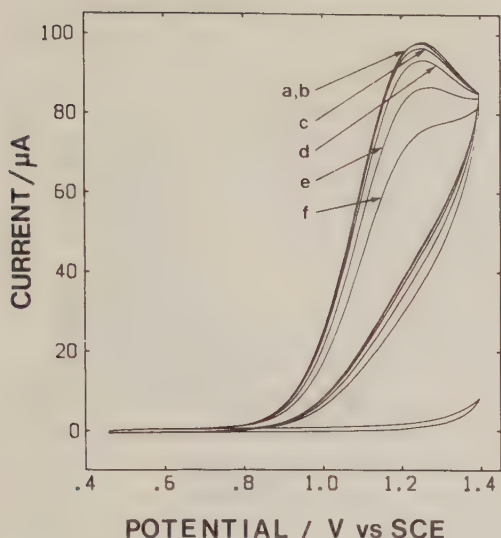


Fig. 2. Cyclic voltammograms of 5 mM H_2O_2 in (a) 0; (b) 0.011; (c) 0.039; (d) 0.093; (e) 0.22; (f) 0.47 M dihydroxyacetone. All solutions were 0.1 M in succinate, pH 5.0. The background current is shown as the bottom plot. Starting potential 0.50 V; sweep rate 50 mV s^{-1} .

quite rapid at higher pH. The formation of methylglyoxal is slow in water and in buffers like glutamate, succinate and pyrophosphate. Other ions, especially phytate and phosphate, act as powerful catalysts [18]. Methylglyoxal can be formed both from dihydroxyacetone and from glyceraldehyde although the latter reaction is slower. Some rate constants are available at 40°C [18] and at 50°C [15] but the data are not sufficient for a calculation of the rate of either formation of methylglyoxal or isomerization during measurement or production of dihydroxyacetone. It may be mentioned, however, that if Riddle and Lorenz's data [18] are applied to a solution containing 1.1 M dihydroxyacetone, 50 mM phosphate, pH 7.4 at 40°C , an initial production rate of methylglyoxal of $65 \times 10^{-3} \text{ moles l}^{-1} \text{ h}^{-1}$ is predicted.

Dihydroxyacetone (1.1 M) was mixed with 10 mM hydrogen peroxide and the mixture was analyzed electrochemically at successive times (Fig. 3). The response decreases with time at first, then it levels out to form a plateau which decreases by 4% during the first hour. Standards run before and after showed that the electrode response remained constant. The initial decrease of response was very fast at pH 5 and 6, but slower in more acidic solutions. The initial decrease of response was also noted at pH 7 and 9, but no clear plateau could be seen. The overall loss of response was very fast at pH 7 with only 4% of the initial amount remaining after 90 min. About 20% remained after the same time in a solution which had a pH of 9. The response decreased with the same percentage and the same relative time course, independently of concentration in the range 1–10 mM hydrogen peroxide. Standard additions produced a step followed by a rapid 10–15% decrease

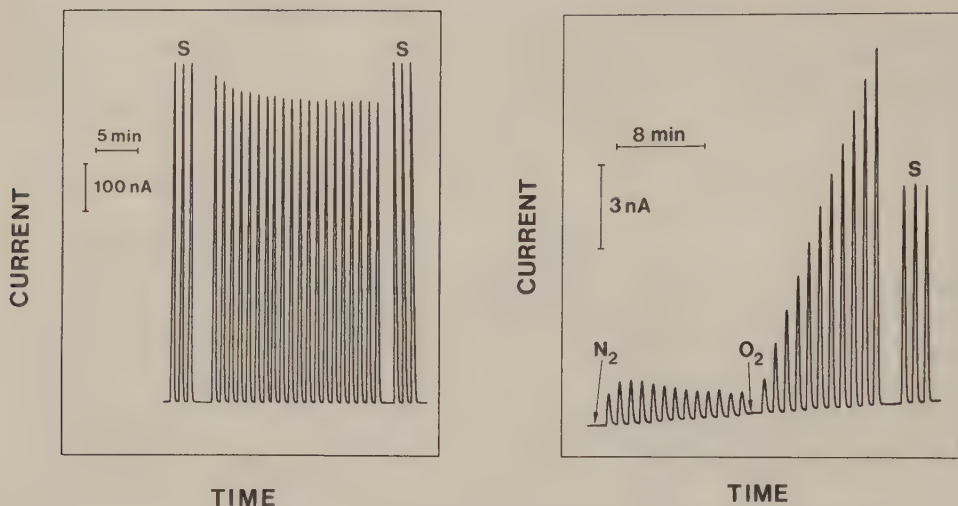


Fig. 3. The time-dependent decrease of the H_2O_2 signal in a solution containing 10 mM H_2O_2 and 1.1 M dihydroxyacetone in 50 mM phosphate buffer, pH 4.0. The S peaks are standards (10 mM H_2O_2) run before and after.

Fig. 4. The effect of oxygen on the peak signal (in 0.11 M dihydroxyacetone in a 0.1 M phosphate buffer, pH 7.0). S is a 0.1 mM H_2O_2 standard.

with the same relative shape as observed initially (pH ≤ 6.0).

The reported behaviour indicates that 10–15% of the hydrogen peroxide is consumed in a side reaction or bound as an adduct or complex which is electrochemically inactive. The possibility that other methods might respond in a different way to this fraction necessitated a comparative study as well as a study of the reactions between hydrogen peroxide and other compounds which are likely to be present in the medium.

About half of the hydrogen peroxide added to a 0.28 M glyceraldehyde solution (pH 4.0) had disappeared after 20 min. The decrease in response was almost exponential. The reaction is thus fairly fast but the formation of aldehyde through isomerization is slow in acidic solutions [15]. The total effect on the peroxide consumption in a dihydroxyacetone solution should therefore be small. There were no other significant reactions except between hydrogen peroxide and methylglyoxal.

Reactions of methylglyoxal

The data given by Riddle and Lorenz [18] show that no methylglyoxal should be produced in the reaction medium at pH 5.0 but that some should be produced in a phosphate buffer at pH 7.0 as mentioned above. Besides the methylglyoxal produced in this way some might be added with dihydroxyacetone as an impurity. It is therefore important to study the interaction between methylglyoxal and hydrogen peroxide.

Hydrogen peroxide was added to a 2.3 mM methylglyoxal solution and samples were analyzed by the electrochemical method at successive times. There was no initial rapid decrease in response such as that described above. There was, however, a slow continuous decrease in response which amounted to about 10% after 30 min at both pH 5 and 7 in a 10 mM peroxide solution. The decrease was slightly faster in a 1 mM peroxide solution. These observations show that it is unlikely that methylglyoxal is involved in the initial rapid decrease of response in the dihydroxyacetone solution.

Reactions of oxygen in dihydroxyacetone solutions

A fresh dihydroxyacetone solution, prepared from deaerated buffer, was bubbled with nitrogen and sampled into the flow injection system. Figure 4 shows that small peaks with time-dependent amplitudes were produced. Part of the response is due to a direct electrochemical oxidation of dihydroxyacetone and the rest to a reaction involving oxygen. Even during the nitrogen bubbling, some oxygen is present from contamination during preparation. When the nitrogen was replaced by oxygen, the analytical system showed successively increased responses. On returning to nitrogen bubbling, the peak heights decreased slowly with time. When the experiment was repeated with a 2.5 mM methylglyoxal solution no influence of oxygen could be seen.

The conclusion to be drawn is that hydrogen peroxide, or a compound with identical effect on the amperometric response, is formed in a reaction involving oxygen. The reaction seems to be reversible as evidenced by the decrease in peak heights during the nitrogen bubbling. It is known that methylglyoxal is produced from dihydroxyacetone via intermediates [17]. A possible explanation is therefore that one of the intermediates forms an adduct with oxygen, which hydrolyzes to hydrogen peroxide and pyruvate or lactate. The presence of small amounts of pyruvate was indeed demonstrated by its reaction with NADH and lactate dehydrogenase. The reaction rate with oxygen at a constant partial pressure and excess of dihydroxyacetone is expected to be constant in agreement with the observed linear increase of the peaks in Fig. 4. Table 2 gives the pH and medium dependence of the reaction, which was found to behave in the same way and to be catalyzed by the same ions as the reactions studied by Riddle and Lorenz [18]. In fact, the rates agreed with those extrapolated from the work of Riddle and Lorenz to within a factor of two. All this leads to the conclusion that hydrogen peroxide is produced spontaneously in the reaction medium if oxygen is present.

Comparison between the electrochemical, enzymatic and calorimetric methods

The amperometric method behaves almost ideally and can be used for almost interference-free determinations of hydrogen peroxide except for one possible deviation i.e., the time-dependent decrease of response in the pres-

TABLE 2

Effect of pH and anions on the production rate of H_2O_2 in a 56 mM dihydroxyacetone solution continuously sparged with oxygen

Medium ^a	pH	H_2O_2 formation (10^{-6} M min ⁻¹)
Water	—	0
Phosphate	2.0	0
Succinate	5.0	0
Phosphate	5.0	0
Pyrophosphate	6.0	0
Phosphate	6.0	1.5
Phosphate	7.0	7.8
Phosphate	8.0	20
Pyrophosphate	9.0	7.2

^a 0.1 M anion.

ence of dihydroxyacetone. It is therefore of interest to investigate whether the observed decrease in response reflects a real decrease in peroxide concentration or if it is an artefact of the method. Because both the enzymatic and the calorimetric methods were available in this laboratory and each measures hydrogen peroxide in a completely different way, it was decided to make measurements in parallel with the three methods.

Table 3 shows a comparison of the amperometric and calorimetric methods when they were used to follow the reaction in a solution containing dihydroxyacetone and hydrogen peroxide. It can be seen that the response decreases to the same degree with both methods. Table 4 gives a comparison of the amperometric and enzymatic methods when both were used to determine the peroxide in a series of solutions containing various amounts of dihydroxyacetone. Again, it can be seen that both methods record a decrease in the peroxide concentration as the dihydroxyacetone concentration increases. Similar results, with agreement between the responses of the two methods, were obtained at other pH values. Both dihydroxyacetone and glycerol produce transients in the spectrophotometer because of changes in the refractive index and this makes the evaluation somewhat more uncertain at low peroxide concentrations.

The enzymatic method was also used to check the mentioned production of peroxide in dihydroxyacetone solutions in the presence of oxygen. The results from the two methods paralleled each other very closely. The rates of peroxide production were found to be 7.8 and 8.1 μM peroxide min⁻¹ with the enzymatic and amperometric methods, respectively. The rates relate to 50 mM dihydroxyacetone, 0.1 M phosphate buffer, pH 7.0. The agreement between the results obtained by the three methods supports the conclusion that each method measures the true hydrogen peroxide concentration even in ageing dihydroxyacetone solutions.

TABLE 3

Comparison of the amperometric and calorimetric methods used to follow the decrease of the H_2O_2 signal with time in a solution containing 10 mM H_2O_2 and 0.55 M dihydroxyacetone in 0.1 M sodium phosphate, pH 7.0

Time of reaction (min)	Relative peak signal (%)	
	Amperometric	Calorimetric
0	100	100
2.5	78	76
6.4	65	65
10.3	55	55

TABLE 4

Concentration of H_2O_2 after 4.0-h incubation as measured by the amperometric and enzymatic methods

(Measurements were made in parallel in solutions initially containing 10 mM H_2O_2 and various amounts of dihydroxyacetone (DHA) in a 0.1 M pyrophosphate buffer, pH 9.0)

DHA conc. (M)	Relative peak signal (%)	
	Amperometric	Enzymatic
0	100	100
0.11	86	89
0.55	40	42
1.1	19	20

If some of the hydrogen peroxide reacts to form organic peroxides, the methods must either be rather insensitive to such peroxides or respond to the same extent. The rate constants for the reaction between some peroxides and peroxidase for the formation of "Compound I" have been evaluated [19] and were found to decrease by one order of magnitude between hydrogen peroxide and methyl peroxide. The rate decreased by another factor of ten for butyl peroxide and still more for branched chain compounds. Peroxidase is known to be completely insensitive to dialkyl peroxides whereas acyl peroxides may react more quickly than hydrogen peroxide. Reactions between hydrogen peroxide and aldehydes are known to produce mono- and di-hydroxyalkyl peroxides. The dihydroxydialkyl peroxides are unstable and decompose rapidly in alkaline media [20].

Even if there is an excess of capacity in the peroxidase reactor of the enzymatic method, it will not be sufficient to give the same response towards organic peroxides with rate constants which are orders of magnitude less than that for hydrogen peroxide. The calorimetric reactor also has excess of capacity but it is insufficient for decomposition of organic peroxides with low reaction rates. Furthermore, there might be differences between the heat produced by decomposing hydrogen peroxide and organic peroxides.

In the amperometric method, the potential of the glassy carbon electrode was selected on the rising part of the oxidation curve in a potential-current diagram in order to reduce the background current [6]. The response will thus be very sensitive to small changes in the oxidation potential of the compounds. The method as designed is thus expected to give either quite different responses to hydrogen peroxide and organic peroxides or to give no response at all to the latter.

Peroxides which equilibrate fairly fast with hydrogen peroxide, and which could therefore be measured via hydrogen peroxide can also be excluded because of the different residence times in the reaction zones. The mean residence times were 40 ms, 1.6 s and 10 s for the amperometric, enzymatic and calorimetric methods, respectively. Unless the equilibration takes <40 ms, the methods should have given different results.

Taken together, these arguments show that it is very unlikely that the response is due to organic peroxides to any significant extent. The electrochemical method described here is therefore well suited for determination of hydrogen peroxide in the biotechnical process mentioned. It is fast and accurate and can easily be adapted to automated sampling. Work is now in progress to use it for computerized control of the hydrogen peroxide concentration in this process.

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REFERENCES

- 1 S.-O. Enfors and B. Mattiasson, in B. Mattiasson (Ed.), *Immobilized Cells and Organelles*, Vol. II, CRC Press, Boca Raton, FL, 1983, Ch. 3.
- 2 M. Ibrahim and H. G. Schlegel, *Biotechnol. Bioeng.*, 22 (1980) 1877.
- 3 J. A. Watson and J. Schubert, *J. Gen. Microbiol.*, 57 (1969) 25.
- 4 F. Lawny, M. Cordonnier and D. Thomas, *A.I.Ch.E. J.*, 21 (1975) 822.
- 5 O. Holst, S.-O. Enfors and B. Mattiasson, *Eur. J. Appl. Microbiol. Biotechnol.*, 14 (1982) 64.
- 6 H. Lundbäck, *Anal. Chim. Acta*, 145 (1983) 189.
- 7 H. H. Weetal, in K. Mosbach (Ed.), *Methods in Enzymology*, Vol. 44, Academic Press, New York, 1976, p. 134.
- 8 B. Olsson and L. Ögren, *Anal. Chim. Acta*, 145 (1983) 87.
- 9 J. M. Reijn, W. E. van der Linden and H. Poppe, *Anal. Chim. Acta*, 126 (1981) 1.
- 10 B. Olsson, L. Ögren and G. Johansson, *Anal. Chim. Acta*, 145 (1983) 101.
- 11 O. Holst, B. Mattiasson, unpublished work.
- 12 B. Danielsson, B. Mattiasson and K. Mosbach, *Appl. Biochem. Bioeng.*, 3 (1981) 97.
- 13 L. Davis, *Bioorg. Chem.*, 2 (1973) 197.
- 14 D. Barham and P. Trinder, *Analyst*, 97 (1972) 142.
- 15 M. Fedoronko and J. Königstein, *Collect. Czech. Chem. Commun.*, 34 (1969) 3881.
- 16 V. Prey, H. Berbalk and E. Steinbauer, *Monatsh. Chem.*, 93 (1962) 237.
- 17 J. C. Speck, Jr., *Adv. Carbohydr. Chem.*, 13 (1958) 63.
- 18 V. Riddle and F. W. Lorenz, *J. Biol. Chem.*, 243 (1968) 2718.
- 19 K. G. Paul, P. I. Ohlsson and S. Wold, *Acta Chem. Scand., Ser B*, 33 (1979) 747.
- 20 B. L. Dunicz, D. D. Perrin and D. W. G. Style, *Trans. Faraday Soc.*, 47 (1951) 1210.

HYDROGEN PEROXIDE AS AN OXYGEN SOURCE FOR IMMOBILIZED Gluconobacter
oxydans CONVERTING GLYCEROL TO DIHYDROXYACETONE.

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Running head: Oxygenation of immobilized cells using hydrogen peroxide

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SUMMARY

A flow injection analysis (FIA) system with amperometric detection was developed for measurement of hydrogen peroxide which was used as an oxygen source for immobilized cells. The analytical capacity was 23 samples per hour. The drift in electrode response was less than 3% over 48 hours. Interference from compounds in the reaction mixture was minimized by dilution of the sample in the FIA system. A constant concentration of peroxide in the reactor was maintained by processing the analytical signal in a computer programmed as a PI-regulator. The regulator actuated a peristaltic pump for dosage of hydrogen peroxide to the reactor. The concentration of dissolved oxygen was followed with a commercial Clark-electrode.

The simultaneous measurements of hydrogen peroxide and dissolved oxygen are discussed with respect to process control.

Conversion of glycerol to dihydroxyacetone by Gluconobacter oxydans immobilized in calcium alginate was used as a model system.

Initial specific productivity increased with increasing hydrogen peroxide concentration. However, decreases in viable counts, enzymatic activities and overall productivities were noted. Various techniques for improving operational stability are discussed.

INTRODUCTION

The supply of oxygen to aerobic organisms is crucial in fermentation technology. This is even more accentuated in systems with immobilized cells (Enfors and Mattiasson 1983), because diffusion limitations are introduced by the matrix (e.g. van Ginkel et al 1983, Sato and Toda 1983, Hiemstra et al 1983) and high cell densities are used in the reactors.

Various solutions to this problem have been investigated. Photolysis of water to oxygen has been tested in a system with algae co-immobilized with bacteria (Adlercreutz et al. 1982). Transport molecules such as hemoglobin have been used in a system with immobilized acetic acid bacteria (Adlercreutz and Mattiasson 1982). In order to increase the solubility of oxygen in the reaction medium biocompatible solvents such as perfluorinated chemicals, have been used (Adlercreutz and Mattiasson 1982). Increased partial pressure of oxygen was used by e.g. Makhotkina et al. (1981). Furthermore, oxygenation by decomposition of hydrogen peroxide has been suggested, and increased productivities have been reported both for immobilized enzymes (Lawny et al. 1975, Hartmeier and Tegge 1979) and for immobilized whole cells (Holst et al. 1982). In the latter case, the hydrogen peroxide was decomposed inside the matrix by catalase of the immobilized cells. Hydrogen peroxide may have deleterious effects on the biocatalyst and a proper control of the supply of the oxygen source is therefore necessary.

Few sensors for metabolites important for bioprocess control are available. Flow injection analysis (FIA) provides a tool for automated chemical analysis on small sample volumes and allows rapid discontinuous measurements (Ruzicka and Hansen 1981).

In this study a flow injection analysis (FIA) system with amperometric detection for determination of hydrogen peroxide concentrations is used for computer controlled additions to a batch reactor with immobilized cells with the aim to maintain a constant concentration of hydrogen peroxide.

The effect of various hydrogen peroxide concentrations on the operational performance has been examined for a system with immobilized aerobic cells. Conversion of glycerol to dihydroxyacetone (DHA) by Gluconobacter oxydans immobilized in calcium alginate was used as a model system.

MATERIALS AND METHODS

Chemicals

Hydrogen peroxide (30%, Perhydrol) and dihydroxyacetone (98%) were obtained from Merck, Darmstadt, FRG. Sodium alginate (type IV) and catalase (EC 1.11.1.6, from bovine liver, 3000 U/ μ l) were purchased from Sigma Chemical Co., USA. All other chemicals were from commercial sources and of p.a. quality.

Immobilized cell systems

Gluconobacter oxydans (ATCC 621) cells were grown and immobilized in beads of calcium alginate gel according to Holst et al. (1982). The mean diameter of the beads was 2 mm. The amount of cells in the beads was 30 mg dry wt./g wet gel. Experiments with the immobilized cells were carried out in 300 mL glass beakers agitated with a magnetic stirrer. Vessels and auxiliary equipment were thoroughly washed with nitric acid (0.1 M) and deionized water, to remove impurities, such as iron, that decomposes hydrogen peroxide.

The reaction medium consisted of 0.5 M glycerol in 0.05 M succinate buffer (pH 5.0) and hydrogen peroxide of various concentrations. Calcium chloride (10 mM) was added in order to stabilize the gel matrix. The reaction temperature was 25°C.

Computer controlled addition of hydrogen peroxide was performed in 150 mL of medium containing alginate beads corresponding to 2 g dry cells per L. Batch experiments at constant concentrations of hydrogen peroxide were run for 20 h.

Measurements of hydrogen peroxide

Hydrogen peroxide was analyzed amperometrically in an automated flow injection analysis (FIA) system. The experimental set up is outlined in Fig. 1. The reaction medium was pumped (2.5 ml min^{-1}) first through a nylon net and then through the injector valve before reentering the reaction vessel. A $5 \text{ }\mu\text{l}$ sample was injected into the carrier stream (1.5 mL min^{-1} of 0.2 M sodium acetate, $\text{pH } 5.0$) with a frequency of 23 per hour utilizing pneumatic actuation. The sample was diluted 100 times in a dispersion coil held at constant temperature. The hydrogen peroxide was oxidized to oxygen and hydrogen ions at a glassy carbon electrode, maintained at $+ 1.2 \text{ V}$ versus Standard Calomel Electrode by potentiostatic control. The current registered by an electronic peak-reader, has been shown to be directly proportional to the hydrogen peroxide concentration (Lundbäck et al 1983). The flow system with the amperometric detector has been described elsewhere (Lundbäck 1983). The electronic system, controlling the fully automated FIA-system, was constructed at the Department of Analytical Chemistry, University of Lund, Sweden. It consists of an analog part including the potentiostat and the peak-reader and a digital part including the sample sequence regulation and the read-out unit.

Measurements of dissolved oxygen and dihydroxyacetone

Dissolved oxygen was monitored with a Clark-electrode (Beckman 0260 Oxygen analyser, Beckman Instr., Inc. Ca., USA). Dihydroxyacetone was assayed as reducing substance using the DNS-method according to Miller et al. (1960). Interference, in the analysis of DHA, from hydrogen peroxide was avoided by adding catalase ($0.5 \text{ }\mu\text{L}/0.5 \text{ ml}$) prior to addition of the DNS reagent.

Determination of viability

For each determination of cell viability 20 gel beads were dissolved in 0.1 M citrate-phosphate buffer (pH 6.0). The dissolution was completed in approximately 15 min. The cell suspensions were diluted and spread on agar plates. The agar contained the culture medium of the bacteria (Holst et al. 1982) and 1.5% agar. Colonies were counted after 48 h incubation at 28°C.

Determination of oxygen consumption rate and catalase activity.

Oxygen consumption rates were estimated in solutions without peroxide by following the decrease in the signal from an oxygen electrode (Beckman 0260 Oxygen analyser, Beckman Instr., Inc. Cal., USA). Measurements were made with fresh beads and after operation for 20 h. The remaining activity after 20 h is expressed in percents of that of fresh beads. The catalase activity was determined by following the decrease in hydrogen peroxide concentration and calculating the rate constants. The remaining activity after 20 h is expressed as ratio between rate constant for beads used 20 h and fresh beads, in percent. All activity measurements were carried out in the medium and at 25°C.

Computer controlled addition of hydrogen peroxide

The peroxide concentration in the reactor was controlled by a PDP11/3 computer, programmed as a PI-regulator. Programs were written in PASCAL. The regulator was tuned manually and the proportional gain in the regulator was set to 0.40 mL/min · mM and the integration time was set to 18 min. Hydrogen peroxide was added with a peristaltic pump (Alitea C-4, Ventur AB, Sweden) actuated by the regulator. The pump was on-off controlled with a maximum flow rate of 0.15 mL min⁻¹. The concentration of the hydrogen peroxide feed was 0.5 M. The computer

system was equipped with facilities for logging of data concerning dissolved oxygen and hydrogen peroxide concentration in the reactor. The feed rate of hydrogen peroxide to the reactor was also logged.

RESULTS AND DISCUSSION

The FIA system for hydrogen peroxide determination has earlier been shown to be accurate and stable in the conversion media containing glycerol, dihydroxyacetone and succinate (Lundbäck et al. 1983). A typical performance of calibration experiments is shown in Fig. 2. The insert gives a calibration curve based on the peaks registered.

The stability of the electrode response was studied in the actual process with immobilized cells converting glycerol to dihydroxyacetone with hydrogen peroxide as an oxygen source. Continuous use of the analytical system for more than 48 hours, corresponding to more than 1000 injections, showed a decrease of 3.0% in the electrode response.

Since hydrogen peroxide is known to be easily decomposed by various impurities (e.g. iron) the equipment used in the experiments was washed with nitric acid (0.1 M) and deionized water. Breakdown of the peroxide in the system was assessed. The decomposition of hydrogen peroxide in medium without alginate beads was followed with the FIA-system during 10 hours. The rate of the decomposition was approximately $50 \mu\text{moles L}^{-1}\text{h}^{-1}$ at an initial peroxide concentration of 1 mM. The rate of decomposition was the same when alginate beads without cells were present in the reactor. The consumption of hydrogen peroxide in presence of alginate immobilized cells was approximately $10 \text{ mmoles L}^{-1}\text{h}^{-1}$ at a constant concentration of 1 mM hydrogen peroxide.

Thus, the decomposition of hydrogen peroxide caused by heterogeneous catalysis or by chemical reactions in the medium is very small.

Oxygenation with hydrogen peroxide can be followed by measuring either oxygen (Ibrahim and Schlegel 1980) or peroxide concentration. In the present study both were measured simultaneously, peroxide using the FIA system and dissolved oxygen using a Clark electrode.

Figure 3 shows experiments in which the pre-set value for the hydrogen peroxide concentration was varied. The time delay for the hydrogen peroxide concentration to reach a new level in the reactor after changing the set-point was approximately 30 min. This may be improved by the use of more sophisticated regulators. Changes in the hydrogen peroxide concentration were accompanied by changes in the dissolved oxygen value. As can be seen in the figure the general appearance of the two curves is almost the same. However, it was noted that after changing the set-point the time required for the dissolved oxygen signal to reach a stable value was somewhat longer.

The correlation between the peroxide and the dissolved oxygen concentrations at various hydrogen peroxide levels was found to be good up to 2 mM of hydrogen peroxide, at which concentration oxygen bubbles begin to appear. These will cause disturbances in the oxygen signal as small oxygen bubbles attach to the electrode membrane. Furthermore, at these concentrations of hydrogen peroxide the decomposition rate of hydrogen peroxide within the beads is higher than the oxygen consumption rate. Oxygen then accumulates in the matrix and eventually disrupt the beads. When operating at peroxide concentrations giving rise to oxygen partial pressures exceeding saturation with air, losses of oxygen will be observed.

These experimental results indicate that it should be possible to use either the hydrogen peroxide or the oxygen signal to control the addition of hydrogen peroxide to the process.

However, by simultaneous measurements of hydrogen peroxide concentration and dissolved oxygen certain advantages are gained.

The yield of dihydroxyacetone, i.e. mol DHA/mol H_2O_2 , produced with hydrogen peroxide as an oxygen source was estimated from 20 hour batch experiments at different concentrations of hydrogen peroxide. The yield was found to be about 1 mol DHA/mol H_2O_2 , when the concentration of hydrogen peroxide was between 0.5 and 1.0 mM.

The correlation between production of DHA and consumption of hydrogen peroxide provides a possibility to control the process at a higher level. Monitoring of the dosage of hydrogen peroxide with time makes it possible to follow the catalase activity and thus the production of oxygen in the process.

Simultaneous monitoring of the dissolved oxygen gives information of the balance between oxygen production and oxygen consumption rates. When the oxygen production rate equals oxygen consumption rate, the dose rate of hydrogen peroxide would reflect the production rate of dihydroxyacetone, which is an indicator of the performance of the process.

The system described above was used for an evaluation of the effects of hydrogen peroxide on the operational performance of a calcium alginate immobilized Gluconobacter oxydans system.

Productivities, survival of cells and enzymatic activities were examined after operation at various hydrogen peroxide concentrations. The initial specific production rate increased with increasing hydrogen peroxide concentration in the range up to 1.5 mM (Fig. 4). The highest specific productivity obtained was 0.7 g DHA/g dry wt $\cdot$ h. This should be compared with the results obtained earlier (Adlercreutz et al. (submitted)) with air as oxygen source when the specific production rate was 0.6 g DHA/g dry wt $\cdot$ h. Although the specific productivity can be increased by the use of pure oxygen, it must be kept in mind that these experiments are carried at low cell densities in the reactor and consequently, if the cell density is increased the oxygen transfer rate from

the gas phase will be the rate limiting step. The mass transfer from gas phase to bulk liquid phase is avoided when hydrogen peroxide is used as an oxygen source.

In order to obtain information on the operational stability of the system, 20 hour experiments at various concentrations of hydrogen peroxide were carried out and the remaining activity of the preparation was determined. The production of dihydroxyacetone with hydrogen peroxide involves two enzymatic steps. Firstly, the hydrogen peroxide is be decomposed to oxygen and water and, secondly, the oxygen should be consumed in the oxidation of glycerol to dihydroxyacetone. It has earlier been shown that the consumption of oxygen reflects the production of dihydroxyacetone (Adlercreutz et al. (submitted)). Consequently, by measuring the oxygen consumption rate and the hydrogen peroxide decomposition rate information about the operational performance can be obtained.

The remaining catalase activity after 20 hours operation at various hydrogen peroxide concentration is seen in Fig. 5. A small decrease in activity is observed when air is used as oxygen source, well in agreement with earlier studies on the operational stability of process (Adlercreutz et al. (submitted)). However, when hydrogen peroxide is used as oxygen source, a drastic decrease in remaining activity is seen. The differences in remaining activity after operation at hydrogen peroxide concentrations in the range 0.5 to 1.0 mM are small. Similar results were obtained when the oxygen consumption rate was measured after 20 hours operation at various hydrogen peroxide concentrations. This is illustrated in Fig. 6, where the remaining oxygen consumption activity is shown as function of the hydrogen peroxide concentration used.

Determination of viable counts gave results confirming the decrease in activity. Viable counts, calculated as colony forming units, decreased to less than 30%, at all hydrogen peroxide concentrations used. Finally, the concentrations of DHA in the reactor were determined after 20 hours operation at various hydrogen peroxide concentrations. The concentration was highest when air was used as oxygen source (21.5 g/L) and lowest when 1 mM hydrogen peroxide was used (6.9 g/L) indicating a decrease in overall productivity.

The decrease in catalase activity, viable counts and dihydroxyacetone production rate when hydrogen peroxide is used as oxygen source indicates an overall destruction of the microorganisms. Further studies on the destruction mechanism are necessary to obtain information that could lead to method for improving the operational stability.

Different techniques to increase the stability already exist. The immobilization as such protects the cells from the bulk concentration of hydrogen peroxide due to formation of a concentration gradient. Free cells are rapidly destroyed by hydrogen peroxide (unpublished results). Co-immobilization with catalase and superoxide dismutase has been used to protect xanthine oxidase (Tramper et al. 1978). Use of mercaptoethanol has been found to decrease the negative effects of hydrogen peroxide produced by methanol oxidase (Couderc and Baratti 1980). Techniques to separate the peroxide from the biocatalysts also exist. Transmembrane catalysis has been suggested (Updike et al. 1973) and the use of hydrogen peroxide to regenerate artificial electron acceptors has been described (Adlercreutz and Mattiasson 1984). Further work along these lines is necessary if hydrogen peroxide should be used as an oxygen source.

The tolerance towards hydrogen peroxide varies with different micro-organisms and the method to improve oxygen supply to biocatalysts described here remains to be tested in other aerobic conversions carried out by immobilized cells.

The use of FIA for control of biotechnical processes as described above is a general approach. Although FIA as a principle is a discontinuous method, the sample throughput can be as high as 60 samples per hour, making FIA a continuous method in a practical sense. When FIA systems are combined with on-line dialysis or filtering devices off reactor measurements can be quite simple.

Therefore , the use of FIA for analysis of metabolites in biotechnical processes offers attractive possibilities. Immobilized enzyme reactors in flow analysis is a well-known concept (e.g. Carr and Bowers 1980, Guilbault 1984). By choosing appropriate enzymes, e.g. oxidases or dehydrogenases, compounds of interest can be converted in the FIA-system to products which are easily detectable.

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REFERENCES:

- Adlercreutz P, Holst O, Mattiasson B (1982) Oxygen supply to immobilized cells: 2. Studies on a co-immobilized algae-bacteria preparation with in situ oxygen generation. Enzyme Microb Technol 4:395 - 400
- Adlercreutz P, Holst O, Mattiasson B (submitted) Characterization of Gluconobacter oxydans immobilized in calcium alginate.
- Adlercreutz P, Mattiasson B (1982) Oxygen supply to immobilized cells: 3. Oxygen supply by hemoglobin or emulsions of perfluorochemicals. Eur J Appl Microbiol Biotechnol 16:165-170
- Adlercreutz P, Mattiasson B (1984) Oxygen supply to immobilized cells: 4. Use of p-benzoquinone as an oxygen substitute. Appl Microbiol Biotechnol 20:296-302.
- Carr PV and Bowers LD (1980) Immobilized enzymes in analytical chemistry, Wiley, New York
- Couderc R, Baratti J (1980) Immobilized yeast cells with methanol oxidase activity: preparation and enzymatic properties. Biotechnol Bioeng 22: 1155-1173.
- Enfors S-O, Mattiasson B (1983) Oxygenation of processes involving immobilized cells. In "Immobilized Cells and Organelles" Vol II:41-60, B. Mattiasson, ed., CRC-Press, Boca Raton, Florida
- Guilbault GG (1984) Analytical uses of immobilized enzymes. Marcel Dekkers, Inc., New York

- Hartmeier W, Tegge G (1979) Versuche zur glucoseoxidation in glucose-fructose-gemischen mittels fixierter glucose-oxidase und katalase. Starch/Stärke 31:348-353
- Hiemstra H, Dijkhuizen L, Harder W (1983) Diffusion of oxygen in alginate gels related to the kinetics of methanol oxidation by immobilized Hansenula polymorpha Cells. Eur J Appl Microbiol Biotechnol 18:189-196
- Holst O, Enfors SO, Mattiasson B (1982) Oxygenation of immobilized cells using hydrogen peroxide; A model study of Gluconobacter oxydans converting glycerol to dihydroxyacetone. Eur J Appl Microbiol Biotechnol 14:64-68
- Ibrahim M, Schlegel HG, (1980) Oxygen supply to bacterial suspensions of high cell densities by hydrogen peroxide. Biotechnol Bioeng 22: 1877-1894
- Lawny F, Cordonnier M, Thomas D (1975) A solution for the oxygen mass transfer problem in immobilized enzyme systems. AIChE Journal 21:822-824
- Lundbäck H (1983) Amperometric determination of hydrogen peroxide in pickling baths for copper and copper alloys by flow injection analysis. Anal Chim Acta 145:189-196

Lundbäck H, Johansson G, Holst O (1983) Determination of hydrogen peroxide for application in aerobic cells systems oxygenated via hydrogen peroxide. Anal Chim Acta 155:47-56

Miller GL, Blum R, Glennon WE, Burton AL (1960) Measurements of carboxymethyl cellulase activity. Anal Biochem 2:127-132

Makhotkina TA, Pomortseva NV, Lomova IE, Nikolaev PI (1981) Glycerol transformations into dihydroxyacetone by polyacrylamide gel immobilized cell of Gluconobacter oxydans. Prikl Biokhim Mikrobiol 17:102-106

Ruzicka J, Hansen EH (1981) Flow injection analysis, John Wiley and Sons, New York

Sato K, Toda K (1983) Oxygen uptake rate of immobilized growing Candida lipolytica. J Ferment Technol 61:239-245

Tramper J, Müller F, Van Der Plas HC (1978) Immobilized xanthine oxidase: kinetics, (in)stability, and stabilization by coimmobilization with superoxide dismutase and catalase. Biotechnol Bioeng 20:1507-1522.

Urdike SJ, Shultz M, Magnuson J (1973) Oxygenation by transmembrane catalysis of hydrogen peroxide. J Appl Physiol 34:271-273

van Ginkel CG, Tramper J, Luyben KChAM, Klapwijk A (1983) Characterization of Nitrosomonas europaea immobilized in calcium alginate. Enzyme Microb Technol 5:297-303

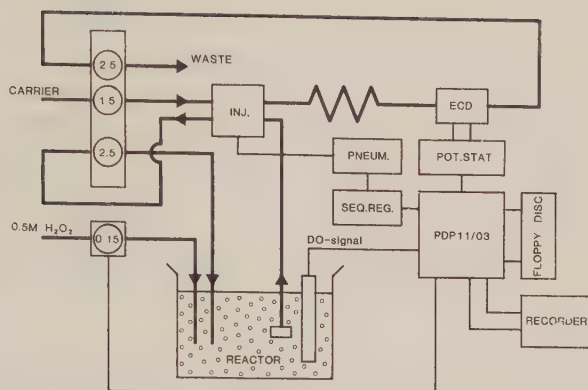


Figure 1. Experimental set-up for computer controlled addition of hydrogen peroxide to a reactor.

Inj. = Injector, ECD = Electrochemical detection, SEQ.REG. = Sequence regulator, PDP11/03 = Computer, POT.STAT. = Potentiostat. Figures in circles are flow rates in mL min^{-1} . The analytical system is described in Materials and Methods.

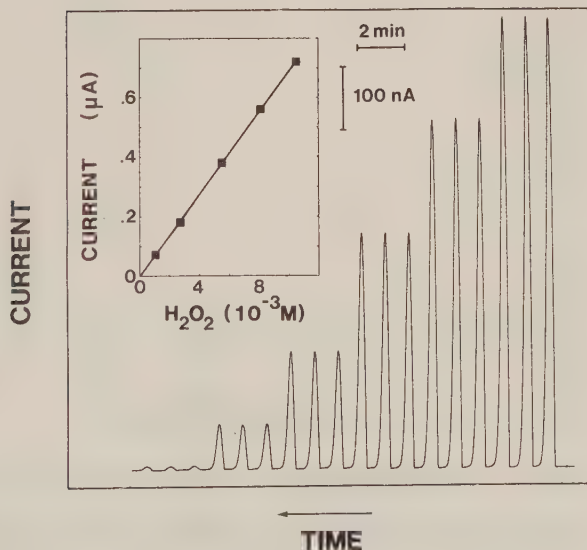


Figure 2. Calibration record and corresponding calibration curve for hydrogen peroxide in a conversion medium analysed in the FIA-system.

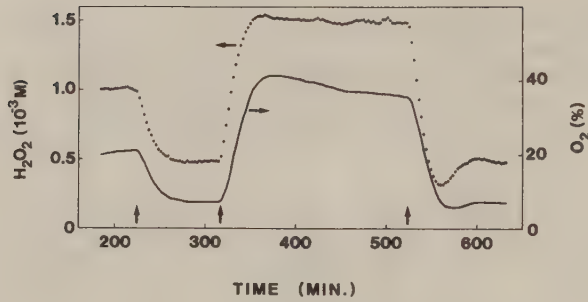


Figure 3. Monitoring of hydrogen peroxide and dissolved oxygen in a batch experiment with immobilized Gluconobacter oxydans converting glycerol to dihydroxyacetone. Vertical arrows indicate step changes of the set point values of hydrogen peroxide concentration.

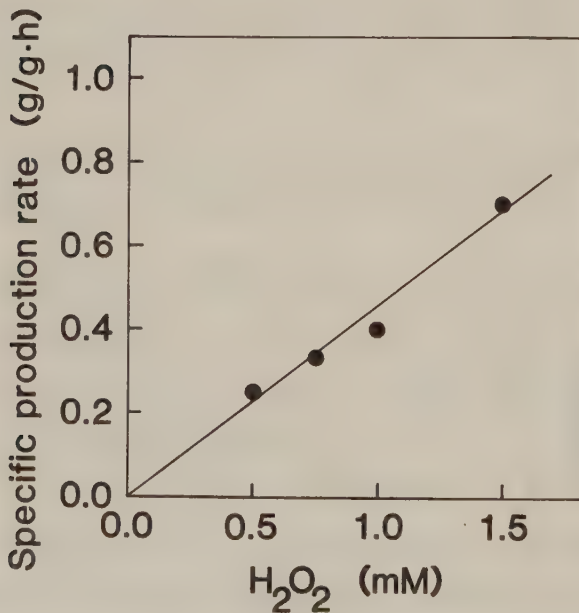


Figure 4. Initial specific production rate of calcium alginate immobilized Gluconobacter oxydans converting glycerol to dihydroxyacetone at various hydrogen peroxide concentrations. Specific production rates are given as g dihydroxyacetone/g dry weight and hour.

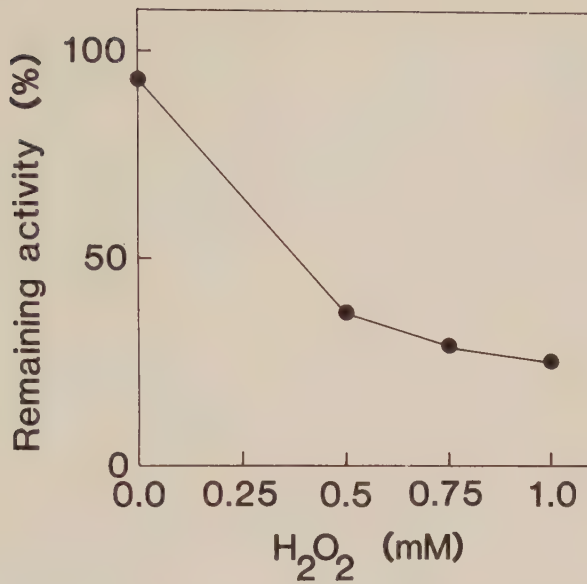


Figure 5. Remaining catalase activity after 20 hours operation at various hydrogen peroxide concentrations. The activities were determined as described in Materials and Methods.

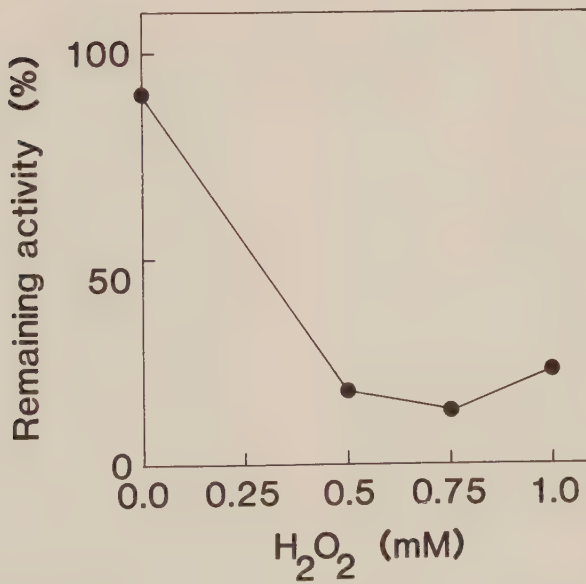


Figure 6. Remaining oxygen consumption activity after 20 hours operation at various hydrogen peroxide concentrations. The activities were determined as described in Materials and Methods.

Oxygen supply to immobilized cells:

2. Studies on a coimmobilized algae–bacteria preparation with *in situ* oxygen generation

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A coimmobilized mixed culture of algae, Chlorella pyrenoidosa, and bacteria, Gluconobacter oxydans, has been studied. The conversion of glycerol to dihydroxyacetone (1,3-dihydroxy-2-propanone), catalysed by the bacteria, was used to indicate the oxygen supply in the immobilized preparation. The oxygen produced by the algae in the coimmobilized preparation was used by the bacteria more effectively than when the cells were immobilized separately and mixed within the reactor. A preparation consisting of only bacteria and no algae was much less effective. The coimmobilized preparation was used in the continuous production of dihydroxyacetone for six days without any significant loss of activity.

Keywords: Symbionts; oxygenation; immobilized *Chlorella*; immobilized *Gluconobacter*; coimmobilization

Introduction

Oxygen supply to dense cell populations is a troublesome problem in conventional fermentation technology, and much work has already been done to get rid of this bottle-neck. Immobilization of cells makes it possible to operate with even higher cell densities than those conventionally used in fermentation processes. Thus, the limitations due to insufficient oxygen supply should be stressed even more in this case. Yet very little has so far been done to improve this unsatisfactory situation. If no new technological methods are developed to solve these problems, several restrictions (such as particle size and cell density) will be encountered when designing an immobilized aerobic cell preparation. Reduced particle size will promote supply of substrates to the interior of the particle and so will a reduction in cell density. On the other hand, some advantages of using immobilized cell preparations, the high catalytic density and the ease of handling of these preparations, will be severely reduced.

Oxygen can be supplied from an external source or be generated *in situ*. A great advantage of the latter method is that mass transfer problems may be markedly reduced. A previous paper reported the use of hydrogen peroxide as an oxygen source.¹ The use of an immobilized preparation containing the oxygen consumer with high catalase [hydrogen-peroxide:hydrogen-peroxide oxidoreductase, EC 1.11.1.6] activity to liberate oxygen from the peroxide

increased the oxidative capacity substantially as compared to when oxygen was supplied with the buffer only.

Another biological approach in the generation of oxygen is the photolysis of water. A preceding paper reported the use of immobilized *Chlorella pyrenoidosa* cells for generating oxygen *in situ*.²

Coimmobilization involves certain advantages over free solution or immobilization in separate particles.^{3,4} Thus it has been shown that, as far as enzymes are concerned, a higher overall rate can be expected when one of the reaction steps is thermodynamically unfavourable, and when both are favourable, the lag period is substantially shorter. Furthermore, pH optima for sequences of catalytic reactions may be different when immobilized.⁵ The present report deals with the coimmobilization of the oxygen generator, *Chlorella pyrenoidosa* and the consumer *Gluconobacter oxydans*, where the latter is used as a general model of an oxygen consumer. *G. oxydans* catalyses the reaction:

glycerol -----> dihydroxyacetone

where oxygen is an acceptor of the hydrogen eliminated.

Experimental

Organisms and cultivation

Chlorella pyrenoidosa (No. 252 in the Collection of Algae at Indiana University)⁶ was grown as described earlier.² *Gluconobacter oxydans* (ATCC 621) was cultivated as previously described.¹ Quantification of cells was done by dry weight determinations.^{1,2}

Part of this work was presented at the IVA-seminar on Biotechnology in Lund, 27 January 1982.

Immobilization

Cells were immobilized in calcium alginate gel as described earlier.² The beads were stored in the dark in buffer solutions containing 10 mM calcium chloride at 4°C until use.

Experimental set-up

The experimental set-up used here consisted of a continuous flow system with a small column reactor (0.55 x 8.5 cm) and is described in detail in the previous paper.² The concentration of dihydroxyacetone was measured instead of the oxygen concentration in the effluent from the column. A 1000 W lamp (Osram SLG 1000) was used as a light source in the optimizing experiments, while fluorescent lamps (LUMA warm white, 4 x 20 W) were used in the long term experiments.

Identification and quantification of dihydroxyacetone

Dihydroxyacetone was identified on high performance thin layer chromatography (h.p.t.l.c.) plates as previously described.¹ Dihydroxyacetone was assayed as a reducing substance using the DNS method of Miller *et al.*⁷

Bio-assay for toxic byproducts of the algae

The following bio-assay was used to ascertain whether the algae *C. pyrenoidosa* produced any toxic byproducts capable of interfering with *G. oxydans*.

A suspension of *G. oxydans* was spread on a Petri dish containing glycerol–agar. The composition of the agar has been described earlier.¹ Filter paper discs with a diameter of 10 mm were placed on the agar and to them was added the medium in which the algae had been cultured, in one set of tests with and in another without algal cells. The tests were run in two parallel sets where one was first incubated in the light (~20 klx) for 2 h directly after application of the incubated pieces of paper to the agar plates. The plates were afterwards incubated in the dark for 46 h at 28°C. Immediately after application of the pieces of paper the other set of plates was placed in the dark incubator for 48 h.

Optimizing experiments

General remarks

Unless otherwise stated, all media contained 10 mM CaCl₂ to stabilize the beads and 30 mM glycerol as a substrate for *Gluconobacter oxydans*. Carbonate (NaHCO₃) was used as a substrate for algal oxygen production. Some different buffer substances were used in different pH intervals (sodium fumarate, maleic acid, succinic acid, Tris). The pH values of the media were adjusted with aqueous NaOH or HCl immediately before use. Unless otherwise stated, the optimizing experiments were made with a flow rate of 0.20 ml min⁻¹.

Preliminary experiments showed that the concentration of dihydroxyacetone in the effluents from the columns became fairly constant after 1–1.5 h. In the optimizing experiments the samples were collected from 1 h 40 min to 2 h after the start of each run. All experiments were carried out at room temperature.

Immobilized preparations used

Three columns (vol. 2 ml), each containing 12 mg (dry weight) immobilized *Gluconobacter oxydans*, were prepared. One of the columns contained *Gluconobacter* only, while the other two columns contained 10 mg (dry weight)

Chlorella pyrenoidosa also. In one of the two columns half of the beads contained bacteria; the other half algae. The two immobilized cell preparations were mixed in the reactor. In the third column the algae and the bacteria were mixed before immobilization, i.e. all beads contained both algae and bacteria.

A medium containing 30 mM carbonate, 30 mM glycerol, 30 mM maleate and 10 mM CaCl₂ (pH 6.0) was pumped through the columns at a flow rate of 0.10 ml min⁻¹. The illumination was 13.8 klx. The rate of dihydroxyacetone production was determined.

Studies on the pH-dependence of the system

Cells of *Gluconobacter oxydans* and *Chlorella pyrenoidosa* were mixed and then immobilized in calcium alginate gel. Columns containing 12 mg (dry weight) bacteria and 17 mg (dry weight) algae per ml reactor volume were prepared. The rate of dihydroxyacetone production in media of varying pH was determined. At each pH value one medium without carbonate and one medium with 30 mM carbonate were tested. The buffers used were 0.1 M fumarate–HCl (pH 4–5), 0.1 M maleic acid–NaOH (pH 6) and 0.1 M Tris–HCl (pH 7–8). The light intensity used was 20.5 klx.

Influence of carbonate concentration on the performance of the whole system

Columns containing the same amounts of cells as in the previous experiment were used. Media containing 30 mM glycerol, 100 mM maleate, 10 mM CaCl₂ and varying concentrations of carbonate (0–100 mM, pH 6.0) were pumped through the columns. The light intensity used was 20.5 klx.

Influence of light intensity

Columns containing the same amounts of cells as in the previous experiments were used. The medium contained 30 mM carbonate, 30 mM glycerol, 100 mM maleate and 10 mM CaCl₂ (pH 6.0). The light intensity was varied between 2.9 and 33.5 klx.

Variation in amount of bacteria

Columns (vol. 2 ml) containing 34 mg (dry weight) algae coimmobilized with varying amounts of bacteria were prepared. A medium containing 30 mM carbonate, 30 mM glycerol, 100 mM maleate and 10 mM CaCl₂ (pH 6.0) was pumped through the columns. The light intensity used was 20.5 klx.

Variation of the amount of algae at constant amount of bacteria

Columns (vol. 2 ml) containing 24 mg bacteria (dry weight) coimmobilized with varying amounts of algae were prepared. A medium containing 30 mM carbonate, 30 mM glycerol, 100 mM maleate and 10 mM CaCl₂ (pH 6.0) was pumped through the columns. The light intensity used was 20.5 klx.

Storage stability

Chlorella pyrenoidosa and *Gluconobacter oxydans* immobilized in calcium alginate were stored in two different buffers, maleate or succinate based, at 4°C in the dark for two weeks. Maleate-based buffer contained 100 mM maleate and 10 mM CaCl₂ (pH 6.0), while succinate based buffer contained 100 mM succinate and 10 mM CaCl₂ and had a pH of 5.0. Samples containing 13 mg algae

and 18 mg bacteria were taken. The samples were packed in columns (vol. 2 ml) and maleate-based buffer supplemented with 30 mM carbonate and 30 mM glycerol was pumped through the columns. The light intensity used was 13.8 klx.

Testing the long-term stability of the coimmobilized system

Two columns (vol. 2 ml) were prepared. One column containing 25 mg (dry weight) immobilized *Gluconobacter oxydans* was used as a blank. The other column also contained 34 mg (dry weight) *Chlorella pyrenoidosa* coimmobilized with the bacteria.

The columns were illuminated by four fluorescent lamps (LUMA 20 W warm white). The distance between the columns and each lamp was 45 mm. A medium containing 5% (v/v) of the culture medium of the algae, 30 mM carbonate, 30 mM glycerol, 100 mM succinate and 20 mM CaCl_2 (pH 5.0) was pumped through the columns at a rate of 0.20 ml min^{-1} . Samples were taken from the effluents of the columns at regular intervals for 6 days.

Results and discussion

Effect of algal products on the bacteria

The bio-assay described above was used to investigate the effect of products of *Chlorella pyrenoidosa* on *Gluconobacter oxydans*. Bacterial growth in the Petri dishes showed no inhibition zones. These results show that, under the conditions used, *Chlorella pyrenoidosa* does not produce any substance that impairs the growth of *Gluconobacter oxydans*.

Effect of coimmobilization

To find out whether the use of algae for oxygen production within the same reactor as an immobilized oxygen consuming organism offered any advantages, three different columns were prepared. One column was prepared with bacteria only (blank); one, with bacteria in half the gel and algae in the other half, with the two preparations mixed in

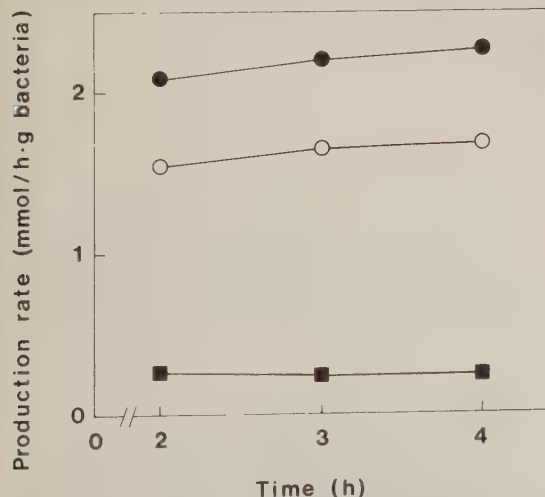


Figure 1 Rate of dihydroxyacetone production of three immobilized cell reactors. One reactor contained *Gluconobacter oxydans* only (■), while the others contained *Chlorella pyrenoidosa* also. The algae were immobilized in separate beads (○) or coimmobilized with the bacteria (●)

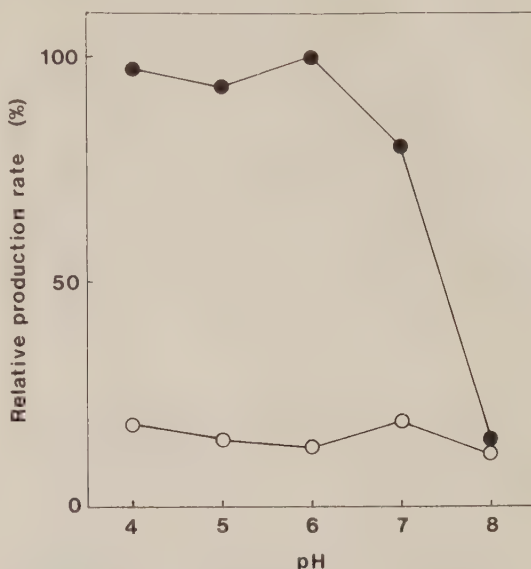


Figure 2 Effect of pH on dihydroxyacetone production rate of coimmobilized *Chlorella pyrenoidosa* and *Gluconobacter oxydans* without carbonate (○) and with 30 mM carbonate (●). Maximal productivity was set at 100%

the reactor; and the third, with gel with coimmobilized algae and bacteria. The column without algae was far less effective than the columns with algae (Figure 1). The column where all the beads contained both algae and bacteria was more effective than the column where some beads contained algae and others, bacteria. This difference is due partly to better utilization of the light when the algae are more widely spread and partly to a more favourable micro-environment. The proximity between the oxygen producer and the oxygen consumer within the beads with coimmobilized cells results in the generation of a favourable micro-environment, where the enhanced oxygen tension around the algae is positively influencing the metabolism of the bacteria. In the rest of the experiments the cells were therefore always mixed before immobilization.

pH-dependence

When no carbonate was used, the rate of dihydroxyacetone production was fairly constant in the pH range 4–8 (Figure 2). A broad optimum around pH 7 was noted. When carbonate was used as substrate for the algae the dihydroxyacetone production rate was rather constant between pH 4 and 6, but it decreased at pH 7 and at pH 8 it was almost as low as without carbonate. This decrease in productivity at higher pH is probably due to the bacteria, which have a pH optimum at 5 and a markedly decreased activity at pH 7–8.¹ The algae have a pH optimum at 7.² The optimal pH for the system studied here was 6, but pH 4 and 5 were almost as good. In the rest of the optimizing experiments pH 6 was used. It should, however, be borne in mind that the pH optimum for the overall reaction is dependent on the ratio between the participating catalytic moieties.⁵

Influence of carbonate concentration

At low carbonate concentrations the dihydroxyacetone production rate increased linearly with the carbonate

concentration (Figure 3). At higher carbonate concentrations the curve levelled off and it reached a maximum at 30 mM. The rather low production rate at carbonate concentration of 100 mM might be due to secondary effects. At this high concentration of carbonate the buffering capacity of the medium was not sufficient with the result that the pH of the effluent from the column was 6.8. Another effect of the high carbonate concentration was a considerable evolution of carbon dioxide, which probably impaired the flow conditions in the column. Furthermore, it must be realized that a high partial pressure of carbon dioxide may be toxic to bacteria.⁸ In the rest of the optimizing experiments a carbonate concentration of 30 mM was used.

Effect of the light intensity on the overall rate

The optimal light intensity was 20.5 klx (Figure 4). The oxygen production by *Chlorella pyrenoidosa* does not show saturation below 33.5 klx.² The decreased dihydroxyacetone production rate at high light intensity is not yet fully understood.

Effect of variation of the amount of bacteria in the reactor

The dihydroxyacetone production rate of columns containing a constant amount of algae and varying amounts of bacteria was measured. When the amount was small the productivity was directly proportional to the dry weight of bacteria in the reactor (Figure 5). However, the curve levelled off and at bacteria densities exceeding 3.5 mg (dry weight) per ml reactor volume no further increase in dihydroxyacetone production rate occurred. This amount of bacteria is apparently sufficient to utilize the oxygen produced by the algae under the conditions used.

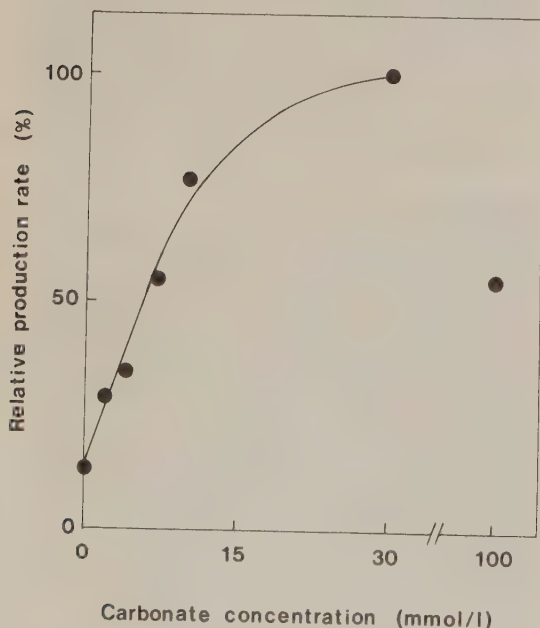


Figure 3 Effect of carbonate concentration on dihydroxyacetone production rate of coimmobilized *Chlorella pyrenoidosa* and *Gluconobacter oxydans*. Maximal productivity was set at 100%

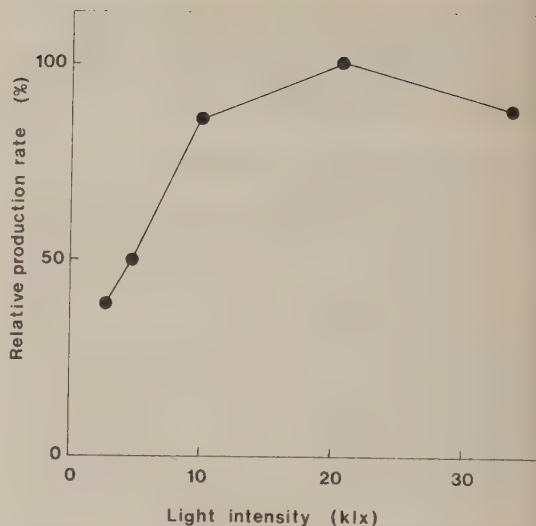


Figure 4 Effect of light intensity on dihydroxyacetone production rate of coimmobilized *Chlorella pyrenoidosa* and *Gluconobacter oxydans*. Maximal productivity was set at 100%

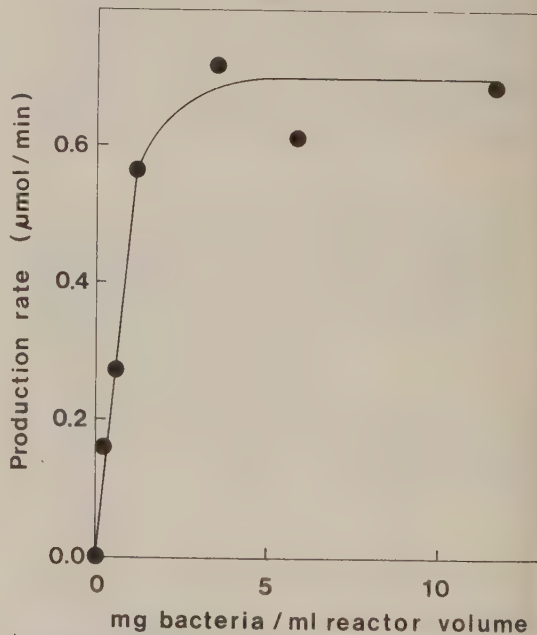


Figure 5 Dihydroxyacetone production rate as a function of the amount of bacteria with a constant amount of algae in a coimmobilized *Chlorella pyrenoidosa*–*Gluconobacter oxydans* preparation

Effect of variation of the amount of algae in the reactor

With a constant amount of bacteria, the dihydroxyacetone production rate increased with the amount of algae up to about 10 mg (dry weight) of algae per ml reactor volume (Figure 6). Further increase of the amount of algae did not increase the production further. This experiment and the preceding one show that in the reactor there are saturation effects with respect to the amount of algae and the amount of bacteria, respectively. Under the conditions

used 10 mg algae ml^{-1} reactor volume utilizes the light as efficiently as possible in this system, and the oxygen produced can be efficiently utilized by 3.5 mg bacteria ml^{-1} reactor volume. These figures will change if the experimental conditions (for example light intensity) are altered. Increasing the cell density further does not give any increased productivity in short term experiments, but it might be beneficial in long term experiments if the specific activity of the cells gradually decreases. Anyway, high cell densities do not lower the productivity of the preparations. In long term experiments reactors containing 17 mg algae and 12.5 mg bacteria per ml reactor volume were used.

Storage stability

Under the conditions used, a maleate-containing buffer at pH 6 is a better storage buffer than a succinate-containing buffer at pH 5 (Figure 7). During the first two days of storage in maleate buffer the activity of the preparation increased. No explanation can be offered for this increase. The number of algal cells did not increase during this period.

The storage stability is quite good. After 14 days in the maleate buffer the activity was higher than immediately after immobilization. In the succinate buffer the activity was fairly constant during the period.

Testing long-term dihydroxyacetone production

In preliminary experiments we tested the long-term stability of the coimmobilized system in the medium found to be optimal in the preceding experiments. However, in this medium the operational stability was rather poor. It was found that the succinate based medium used in the experiments on storage stability (supplemented with 30 mM carbonate and 30 mM glycerol) gave a somewhat better stability of the preparation, although the initial activity was lower than that in the optimal medium. Succinate based media at pH 5 was used in the rest of the long-term experiments.

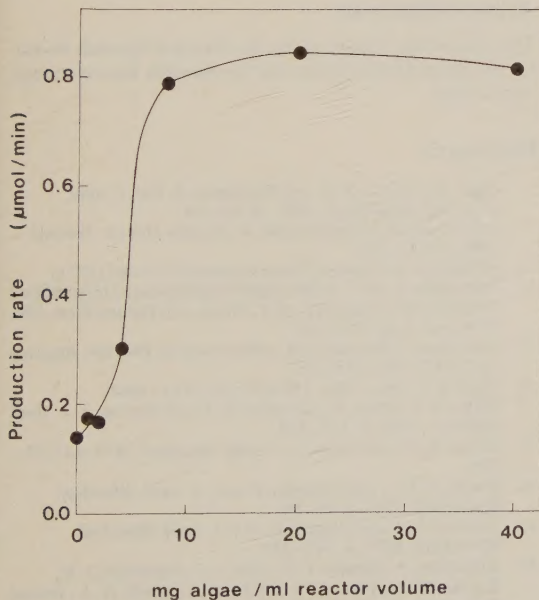


Figure 6 Dihydroxyacetone production rate as a function of the amount of algae with a constant amount of bacteria in a coimmobilized *Chlorella pyrenoidosa*—*Gluconobacter oxydans* preparation

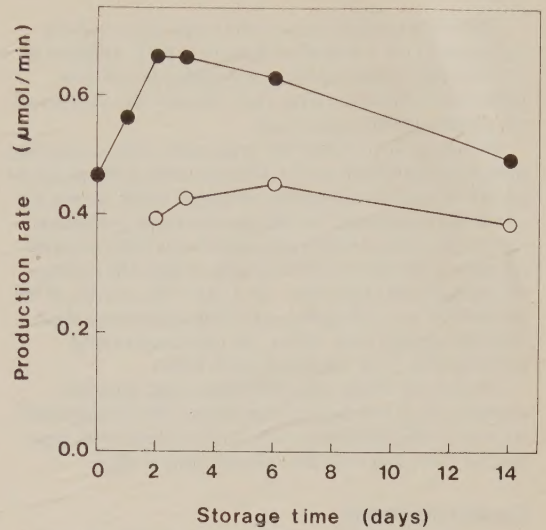


Figure 7 Storage stability of a coimmobilized preparation of *Chlorella pyrenoidosa* and *Gluconobacter oxydans* in a maleate based buffer with pH 6 (●) and in a succinate based buffer with pH 5 (○)

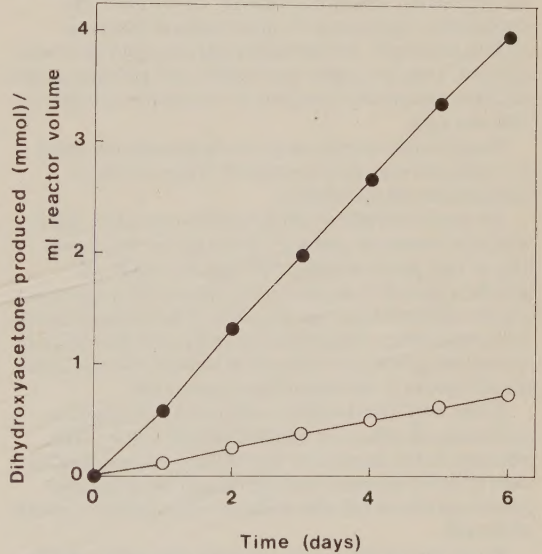


Figure 8 Total amount of dihydroxyacetone produced in a column containing immobilized *Gluconobacter oxydans* cells (○) and a column containing the same amount of bacteria coimmobilized with *Chlorella pyrenoidosa* cells (●)

It was assumed that the decrease in productivity was due to the algae of the preparation. In an effort to reactivate the algae the preparation was treated for short periods (30–40 min) with the culture medium of the algae. With such reactivation the activity of immobilized *Chlorella pyrenoidosa* without bacteria could be maintained for one month.² This treatment increased the dihydroxyacetone production rate. However, the results were even better when a little of the culture medium was added to the production medium. As the culture medium contained phosphate, magnesium and other ions that destabilize the calcium alginate gel, the concentration of calcium chloride in the medium was raised to 20 mM.

With these modifications of the production medium (succinate at pH 5 instead of maleate at pH 6, addition of 5% (v/v) algal culture medium and raising the calcium chloride concentration from 10 to 20 mM), the stability of the preparation was quite good.

During the first 24 h of the experiment, the productivity of the coimmobilized preparation increased, perhaps due to growth of algae on the surface of the gel beads. During the rest of the experiment the dihydroxyacetone production rate of the coimmobilized preparation was fairly constant (Figure 8). Owing to intense growth of algae the column was plugged after operation for 6 days. The activity of the preparation was still quite good. If the superfluous algae had been washed away earlier, the experiment could probably have been continued much longer.

The activity of the column without algae showed a constant activity during the experiment. The accumulated amount of dihydroxyacetone from the column with algae was 5.4 times that from the column without algae.

General discussion

The use of coimmobilized microorganisms is a special variant of mixed cultures. They possess unique metabolic potentials in the sense that a mixture of organisms with complementary metabolic pathways can be used. The mechanisms regulating such mixed cultures have been studied intensively and several general principles have been revealed. These principles deal mainly with growing cultures and thus concern the regulation of the relative numbers of different cells.

Those studies contain much useful information about e.g. ratios between participating cell species in the co-immobilized cell preparation.

An ideal immobilized cell preparation would be one in which the metabolic pathways necessary for the conversion to take place operate at full capacity, while cell growth is limited to an absolute minimum. Such a situation is quite different from that known in mixed culture fermentation technology. It can therefore be foreseen that different criteria will govern the interactions between the participating cell species in the immobilized preparation.

So far, very little has been done in the area of micro-environmental effects on immobilized cell species. With reference to the situation in immobilized enzyme preparations it can be expected that the changed microenvironmental conditions will also influence the metabolic activity of the cell.

To our knowledge this report is the first where coimmobilized cell preparations have successfully been designed to create a favourable microenvironment and thus a good multistep catalyst. Perlman and coworkers⁹ tried this approach in their effort to produce 2-keto-L-gulonid acid, but found separate immobilization to be superior to coimmobilization.

When immobilizing a mixed culture, very high cell densities may be maintained, in some cases higher than what is practically feasible in free solution. Furthermore, the physiological conditions of immobilized cells might have changed from those in free solution. Some indications point towards a lower growth rate and a higher productivity for which reason one can assume that the rules for regulation between the different species in a coimmobilized preparation differ from those in free solution.¹⁰

As mentioned earlier, the microenvironment created in the solid phase differs from that in the bulk phase. These

differences may be ascribed to the matrix itself, but also to enrichment of metabolites from the entrapped cells. The importance of such an environment for immobilized enzymes has been studied and explained in some detail but not for immobilized cells.

As stated earlier² immobilization in alginate is not the best choice for this application.

Use of photosynthetic reactions for oxygen generation places very specific demands on the design of the reactor.

Symbionts of the type discussed in this paper are special cases of mixed cultures, in that the two participating cell species are often coordinated in more than one way. In the natural symbionts, e.g. lichens an integrated metabolic pattern is known to exist with specific functions for each of the participating cell species. Such a degree of integration is not expected in an artificial symbiont, but interactions involving more than one or a few common intermediates may be expected.

The interaction may be positive as well as negative. *Chlorella* is known to generate toxic peroxides of lipids^{11,12} but no evidence of any effect of such substances was seen in the present study. *Gluconobacter* must in this context be regarded as a model system to test the effect of coimmobilized green algae. In future applications it may be of interest to use oxygen consumers which produce carbon dioxide and thereby create possibilities for a favourable microenvironment for both metabolic entities.

The use of algae to generate oxygen within immobilized cell systems offers certain advantages. Thanks to the *in situ* generation, it is possible to maintain a uniform oxygen tension also in packed bed reactors. This was earlier found to be a problem when hydrogen peroxide was used as an oxygen source.¹ Furthermore, since oxygen in too high a concentration may be toxic it should be stressed that the use of photolysis provides a possibility to control the oxygen tension by regulating the light intensity.

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References

- Holst, O., Enfors, S.-O. and Mattiasson, B. *Eur. J. Appl. Microbiol. Biotechnol.* 1982, **14**, 64–68
- Adlercreutz, P. and Mattiasson, B. *Enzyme Microb. Technol.* 1982, **4**, 332–336
- Mattiasson, B. *Doctoral Thesis* University of Lund (1974)
- Mattiasson, B. 1977. in *Biomedical Applications of Immobilized Enzymes and Proteins* (T. M. S. Chang, ed.) Plenum Press, New York, vol. 2, pp. 253–269
- Gestrelus, S., Mattiasson, B. and Mosbach, K. *Biochim. Biophys. Acta* 1972, **276**, 339–343
- Starr, R. C. *Am. J. Bot.* 1964, **51** (9), 1013–1044
- Miller, G. L., Blum, R., Glennon, W. E. and Burton, A. L. *Anal. Biochem.* 1960, **2**, 127–132
- Enfors, S.-O. and Molin, G. *J. Appl. Bacteriol.* 1978, **45**, 279–285
- Martin, C. K. A. and Perlman, D. *Eur. J. Appl. Microbiol. Biotechnol.* 1976, **3**, 91–95
- Navarro, J. M. and Durand, G. *Eur. J. Appl. Microbiol. Biotechnol.* 1977, **4**, 243–254
- Robertson, P., Daniels, T. C., Eiler, J. J., Gunnison, J. B., Kumer, W. D., Oneto, J. F., Strait, L. A., Spoer, H. A., Hardin, G. J., Milner, H. W., Smith, J. H. C. and Strain, H. H. *Science* 1944, **99**, 351–352
- Spoer, H. A., Smith, J. H. C., Strain, H. H., Milner, H. W. and Hardin, G. J. *Carnegie Inst. Wash. Pub. No 586*, 1949

